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Can Evolution of Gut Microbiota alter *C. elegans* Longevity?

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Sumário

Na natureza, os organismos interagem entre si e com o seu ambiente, criando assim interacções ecológicas que influenciam processos evolutivos. Este tipo de interacções tem vindo a ser descrito e estudado como *feedbacks* entre processos ecológicos e evolutivos. Um exemplo particular é a interacção entre hospedeiros e microorganismos, uma vez que os microorganismos são fundamentais para a fisiologia e ecologia dos seus hospedeiros. Dentro da comunidade microbiológica, o microbiota intestinal tem sido largamente estudado uma vez que pode influenciar o desenvolvimento embrionário, bem como a saúde em indivíduos adultos ao providenciar nutrientes e metabolizar compostos não digestíveis.

A interacção entre hospedeiro e microbiota intestinal pode ser estudada em organismos modelo, como é o caso de *C. elegans*. Este nemátode tem sido reconhecido como fundamental para ecologia dos solos e as suas bactérias intestinais influenciam processos como resposta imunitária, fecundidade e longevidade. Na natureza, o microbiota de *C. elegans* é bastante complexo, sendo composto por diversas espécies de bactérias. Contudo, em ambiente laboratorial o microbiota do nemátode pode ser estudado com uma única espécie de bactéria.

Diversos estudos têm abordado a interacção entre *C. elegans* e bactérias intestinais. Hoje em dia sabe-se que diferentes estirpes de bactérias influenciam de modo diferente o seu hospedeiro, nomeadamente a sua longevidade. Adicionalmente, a relação do nemátode com as bactérias intestinais muda ao longo da vida do mesmo, passando de uma relação predador-presa para uma relação hospedeiro-agente patogénico. Esta alteração na interacção dos dois organismos está directamente relacionada com a acumulação de bactérias no intestino, que por sua vez está directamente relacionada com níveis de patogenicidade.

Para além deste efeito de acumulação bacteriana, as bactérias também podem influenciar a longevidade dos organismos através de outros mecanismos, nomeadamente existem diversas bactérias que são patogénicas para *C. elegans*. Um exemplo é a bactéria *Serratia marcescens*, conhecida por ser patogénica oportunista para humanos e patogénica para o nemátode. O estudo de bactérias patogénicas como microbiota intestinal em *C. elegans* é bastante vantajoso, uma vez que estas são capazes de colonizar o intestino do nemátode.

Atendendo a esta interacção entre hospedeiro e bactérias patogénicas, o principal objectivo deste trabalho foi investigar se a evolução do microbiota intestinal está directamente relacionada com alterações no hospedeiro, nomeadamente, alterações na longevidade do mesmo. Para tal, foi utilizado um protocolo de evolução experimental onde o microbiota intestinal do nemátode foi seleccionado através da longevidade de *C. elegans*.

De modo a seleccionar microbiota intestinal capaz de alterar a longevidade do hospedeiro, este foi seleccionado em diferentes intervalos de tempo, o que permite seleccionar para menor ou maior longevidade.

Adicionalmente, foram utilizadas duas estirpes diferentes de *C. elegans*, a N2 (*wild-type*) e a mutante derivada PS3551 que possui uma mutação no gene *hsf-1*. Este gene está relacionado com respostas a stress e tem sido associado a alterações na longevidade de *C. elegans*, uma vez que a perda de função deste gene leva a uma diminuição da longevidade.

Neste trabalho foi também desenvolvido um novo protocolo que permite criar condições experimentais de modo a testar o impacto de diferentes níveis de selecção na evolução de bactérias, uma vez que a selecção pode actuar em diferentes níveis biológicos. Estudos anteriores demonstraram a importância de uma análise com diferentes níveis de selecção na interpretação de certos processos evolutivos, nomeadamente na evolução de organismos patogénicos.

Por fim, neste trabalho é apresentado um novo protocolo que permite transferir nemátodes adultos de forma muito rápida e com pouco esforço. Neste protocolo, os indivíduos são transferidos com um filtro que permite separar os adultos de ovos e larvas e transferir cerca de 300 indivíduos de uma só vez. Isto permite reduzir o tempo dedicado à transferência de indivíduos, possibilitando o aumento do número de populações experimentais e consequentemente o número de condições experimentais em teste.

Os nossos resultados indicam que a selecção indirecta do microbiota intestinal influencia de modo claro a longevidade do hospedeiro, uma vez que após apenas um ciclo de selecção, nemátodes expostos a microbiota seleccionado tinham uma maior longevidade. Até à data, a evolução do microbiota intestinal através da selecção de uma característica do hospedeiro nunca tinha sido aplicada num hospedeiro animal e os nossos resultados abrem a porta para a possibilidade de seleccionar o microbiota de modo a melhor as características do hospedeiro. Apesar de ter sido demonstrado que a longevidade de *C. elegans* é alterada em função da selecção do seu microbiota, não foi possível seleccionar diferentes bactérias capazes de diminuir ou aumentar a longevidade. Contudo, este resultado provavelmente deve-se ao facto de se ter feito apenas um ciclo de selecção e provavelmente com mais ciclos de selecção será possível seleccionar diferentes bactérias, uma vez que se sabe que existem estirpes específicas de bactérias capazes de aumentar ou diminuir a longevidade de *C. elegans*.

Os nossos resultados também demonstram que o protocolo utilizado para criar diferentes condições experimentais com diferentes níveis de selecção funciona e que esta abordagem é importante para a evolução das bactérias. De facto, o aumento na longevidade de *C. elegans* foi apenas visto quando os animais se encontravam numa condição em que a selecção individual a nível das bactérias é menor.

O aumento de longevidade está relacionado com o facto de se estar a seleccionar uma bactéria menos patogénica, o que só é possível quando a selecção individual é reduzida, uma vez que em competição directa uma bactéria mais patogénica deverá ter um maior *fitness*. Uma vez mais, estes resultados mostram que a evolução da interacção entre hospedeiro e microbiota do intestino é um processo complexo e futuros estudos poderão ter em consideração diferentes níveis de selecção.

Neste trabalho, também foi demonstrado que a competição entre bactérias pode, por si só, influenciar a longevidade de *C. elegans*, uma vez que a longevidade do nemátode quando exposto a uma única estirpe bacteriana é diferente da longevidade quando expostos a uma mistura das mesmas estirpes. Este resultado é bastante interessante tendo em conta que na natureza o microbiota de *C. elegans* não é composto por apenas uma espécie de bactéria, mas sim por diversas espécies que poderão competir entre si.

Finalmente, *C. elegans* expostos a bactérias com novas resistências a antibióticos têm uma maior longevidade, o que evidencia a importância da resistência a antibióticos no estudo da interacção entre hospedeiros e microorganismos.

Palavras-chave: Interacções hospedeiro-agente patogénico; Microbiota intestinal; Longevidade; Evolução experimental; Níveis de selecção.

Abstract

In nature, organisms interact with each other and with their environment, thus influencing evolutionary processes. One of the most prevalent ecological interactions is the relationship between host and microorganisms, namely the gut microbiota. This kind of interaction can be studied in model organisms such as *C. elegans*, since the nematode has been acknowledged as a major player in soil ecology and the worms' gut bacteria can influence processes such as immune response, fecundity and longevity. *C. elegans* gut microbiota studies can be done with single bacterial species. The possibility of using pathogenic bacteria capable of colonizing the gut, such as *S. marcescens*, provides an excellent model to study host-microorganism interactions. Given this interplay between host and pathogenic bacteria, we aimed to investigate if gut microbiota evolution is directly related with changes in the host, particularly its longevity. For that, the worms' gut microbiota was indirectly selected via *C. elegans'* longevity. Moreover, we developed a protocol to create experimental conditions that allow us to test the impact of different levels of selection, since this theoretical perspective can be relevant in explaining the evolutionary processes of pathogen evolution. Our results show that by selecting gut microbiota it is possible to alter host longevity, which has never been reported in an animal host. Moreover, results indicate that our protocol creates different environments which allow for different levels of selection, which in turn are important for bacterial evolution. In fact, an increment in longevity was only seen when bacterial individual selection was minimized. In this work, we also found that bacterial competition can have an impact on *C. elegans'* longevity, which is interesting since in nature worms are exposed to diverse bacteria that can compete with each other. Finally, we found that the gain of antibiotic resistance can influence the interaction between host and microorganisms.

Keywords: Host-pathogen interaction; Gut microbiota; Longevity; Experimental evolution; Levels of selection.

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I. Introduction

1. Species interactions

In nature, organisms are part of ecosystems where one species co-occurs and interacts with other species along with the environment, creating a network of biotic and abiotic interactions[1]. The interplay between natural selection and ecology has been gaining relevance, giving rise to the Eco-Evolutionary Feedback field[2,3]. In light of this concept, selection mediated by ecological dynamics drives evolutionary changes which in turn alter the form of ecological interactions[3]. For instance, bacterial species adapting to a new abiotic environment had enhanced growth rate when in polyculture, highlighting the impact species interactions has on adaption to a novel environment[4].

Taking into consideration that an ecosystem usually has an exceptionally complex network, eco-evolutionary feedbacks can be studied as relationships between one or more species and their environment. A particular case is the interaction between hosts and microorganisms, which could be seen as an ecological community[5]. Healthy animals and plants are colonized by a large and diverse number of microorganisms[5] where bacteria are considered key players in multiple aspects of organisms' biology[1]. Additionally, previous works have shown that host-parasite interactions can lead to extremely rapid evolutionary changes[6]. One major focus of host-microorganism interactions is the relationship between a host and its gut microbiota.

2. Host-gut microbiota interactions

The intestinal tract of animals is a favorable niche for microorganisms where these form a complex and dynamic community that can be seen as an ecosystem[5]. It is estimated that the human gut microbiota includes 10^{14} bacteria[7], which is about the number of stars in 1000 Milky Way galaxies.

In recent years, the importance of gut bacteria in host health has gained relevance since bacteria were seen to affect both development and adult-health by acting as stimuli for morphogenesis or by providing essential nutrients and metabolizing indigestible compounds[8]. Indeed, 10% of the metabolites in the mammalian blood flow are from bacterial origin[9].

Gut epithelia are constantly exposed to hostile bacteria[10] and resident microbes can provide strong protection against opportunistic pathogens[11]. This protection can be mediated by producing toxins, limiting resource availability or by directly modulating host immune response[8,12].

Alternatively, a disruption in the gut microbiota could result in pathological outcomes, such as obesity, diabetes, autoimmune disorders, inflammatory bowel disease or even some forms of cancer[13,14].

Besides its importance for host health, the large population size and short generation time of microbes create the potential for rapid adaptation, making the gut microbiota the perfect model for eco-evolutionary studies.

However, the number and nature of questions that can be answered by directly studying mammalian gut microbiota are limited. For instance, host generation time restricts experimental evolution assays concerning host-gut microbiota interactions. A possible solution is to use model organisms, such as *Caenorhabditis elegans*. *C. elegans* is an excellent model organism to study gut microbiota interactions, given that the intestine is their largest somatic organ and it is typically full of microbes[13]. Moreover, the gut microbiota in *C. elegans* performs many of the same functions of human gut microbiota[13] (Figure 1).

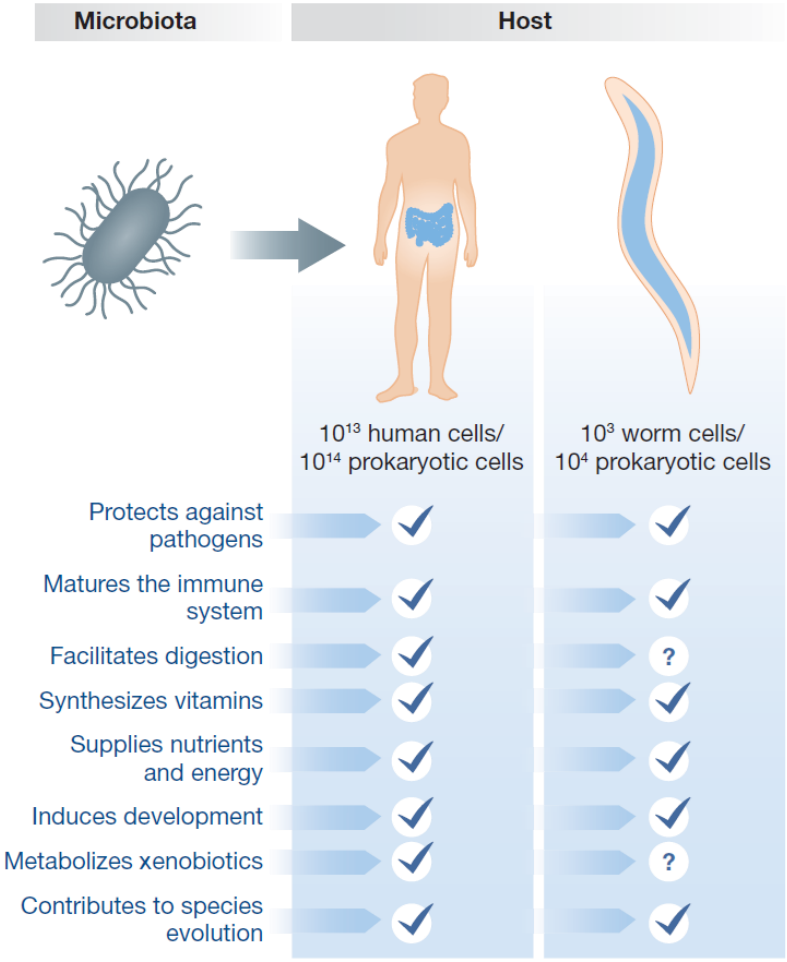

Figure 1 – Functions of gut microbiota in human and nematode hosts.

Effects of intestinal microbiota on the host.

Adapted from Cabreiro *et al*., 2013.

3. *C. elegans* as a model organism

The nematode *Caenorhabditis elegans* was established as a model organism by Sydney Brenner in 1974[16]. Today, *C. elegans* is a very well established model in several fields such as development, (innate) immunity, apoptosis and aging[16].

C. elegans is a small free-living nematode that is found in rotting fruit, vegetation and soils of temperate regions[17,18]. Populations are mainly formed by self-fertilizing hermaphrodites and a small percentage of males[18]. The worm has a short generation time (4 days), which includes 4 larval stages and a large progeny – each hermaphrodite can lay between 250-300 eggs[17].

In laboratory conditions, *C. elegans* normally grows at 20-25°C on solid agar medium and is easily maintained in monoxenic cultures of *Escherichia coli*, strain OP50 (an uracil auxotroph bacteria derivative of *E. coli* B) [15]. Under those conditions, wild-type *C. elegans* has a lifespan of 2-3 weeks[17,18]. Furthermore, worms can be frozen and they are fully transparent, allowing the use of fluorescent reporters in living animals[17]. *C. elegans* also present a powerful set of genetics tools, with approximately 3000 different mutants strains publicly available and its whole genome sequenced[17].

Finally, the worm possesses evolutionarily conserved signaling pathways for innate immunity, such as the insulin/IGF signaling pathway, the p38 MAPK and the transforming growth factor (TGF-β) [13,18,19].

4. *C. elegans* and its gut microbiota

The nematode has been recognized as a major player in soil ecology, where it encounters a wide variety of different bacteria[20]. The interaction between *C. elegans* and gut bacteria is very complex and changes throughout the worm's life course (Figure 2)[15]. Accordingly, during development their interaction with bacteria is exclusively predatory and bacteria found within the intestinal lumen are never intact[15]. In contrast, live bacteria can only be seen in young adults (day 4-5 of adulthood) where they can form symbiotic or commensal communities in the gut[15]. As worms age, they are more sensitive to bacterial infections and bacteria proliferation within the intestinal lumen becomes detrimental to the host[21].

This kind of interaction has also been reported in *Drosophila*, since the presence of bacteria during the first week of adulthood enhances longevity and in the last stage of life causes a decrease in lifespan[21].

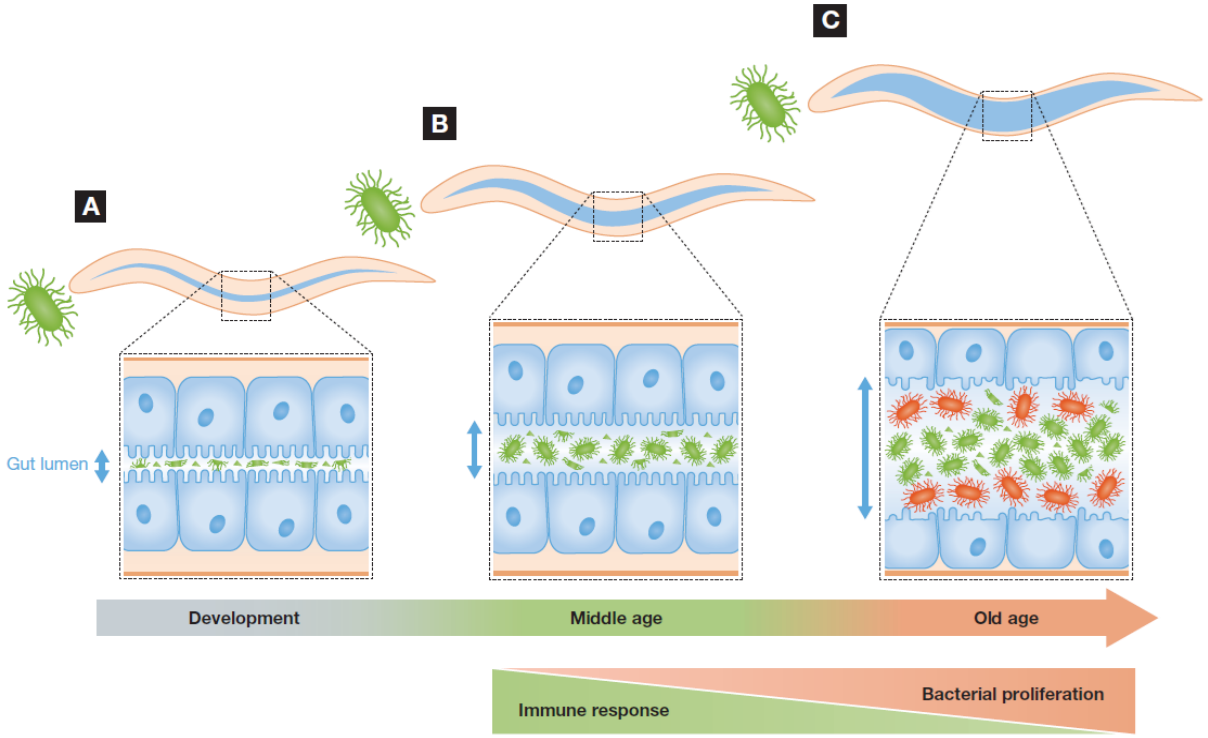

Figure 2 – Changes in *C. elegans*–gut microbiota interactions during the course of life.

A – During development, bacteria serve as a source of food;

B – In young adults, bacteria escape the grinders action and establish a community in the intestinal lumen;

C – As the worm ages, bacterial accumulation within the lumen become detrimental to the host.

From Cabreiro *et al*., 2013

4.1 Bacteria as a food source

As mentioned above, bacteria serve exclusively as a food source in the initial phases of *C. elegans* life (Figure 2A). When *C. elegans* feed, bacteria pass through the pharynx to the terminal bulb, where there is an organ that destroys bacterial cells. This organ – the grinder – is a tripartite array of interlocking tooth-like structures that process all food[15,22] (Figure 3). After passing through the pharynx, bacterial cells reach the intestine. The worm intestine is a nonrenewable monolayer of 20 epithelial cell arranged to form a tube with a central lumen[15].

The quantity of live bacteria in the nematode intestine is influenced by pharyngeal pumping rate, grinder integrity and digestive system efficiency[23]. Additionally, host immune system and bacterial proliferative capacity are also important features in controlling gut microbiota[13]. Peculiarly, despite the fact that *C. elegans* kills bacteria, the worm has a nutritional requirement for live, metabolically active bacteria, since animals fed on non-viable bacteria appear ill and have diminished fecundity[24].

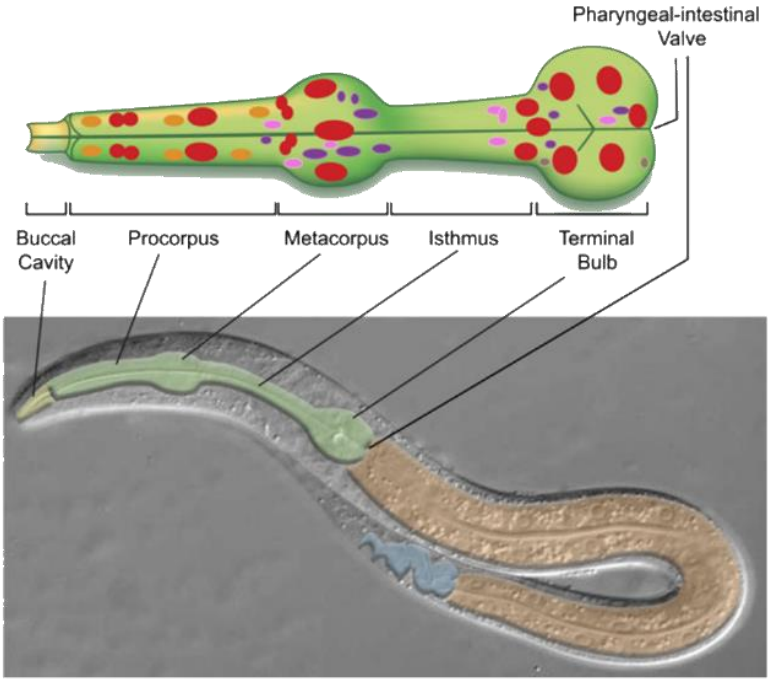

Figure 3 – *C. elegans* digestive tract.

The digestive tract is an epithelial tube consisting of the buccal cavity (yellow), pharynx (green) intestine (orange) and hindgut (blue).

From WormBook

4.2 C. elegans core gut microbiota

In young adults, it is possible to see bacterial accumulation and proliferation inside *C. elegans* intestinal lumen[25]. During young adulthood, bacteria can form a symbiotic or commensal community within the intestine (Figure 2B). In fact, recent studies have reported that *C. elegans* possesses a species-rich core gut microbiota dominated by Proteobacteria phyla, namely the Enterobacteriaceae family and the *Pseudomonas* genera[1,8,26]. In addition, *C. elegans* microbiome is distinct from its substrate environment and at least from the congeneric *C. remanei*[8,26]. A core gut microbiota has also be described in apes, zebrafish, termites, bees and *Drosophila*[7] (where the core microbiota varies between and within different species, habitats and even laboratories)[27].

4.3 Bacteria accumulation is deleterious for C. elegans

Even though a healthy core microbiota has been described for *C. elegans*, as the worm ages they lose the capacity to control the number of live bacteria that reach the intestine. This loss is related with a decline in intestinal functions, such as ingestion and defecation[29] as well as with a decline in innate immunity[28]. In this phase, even non-pathogenic bacteria like *E. coli* OP50 are able to escape grinder function and reach the intestine alive, where they become opportunistic[25].

Accumulation of undigested bacteria is associated with symptoms of pathology, which include increased variability in intestinal shape and size and distention of the pharynx and intestinal lumen[30] (Figure 2C). In addition to the morphological changes, bacteria accumulation within the gut has been correlated with higher mortality rates, since *C. elegans* grown on bacteria unable to proliferate had a longer lifespan[30]. In fact, an inverse correlation between bacterial accumulation and *C. elegans* lifespan was recently described, which suggests that bacteria accumulation contributes to aging[25,31].

However, bacterial accumulation is not the only factor that contributes to increased mortality. Grinder-defect mutants only show increased mortality associated with a compromised immune system[31] and bacteria such as *Enteroccocus faecium* are capable of colonizing the gut without causing significant mortality[32]. This suggest that non-pathogenic bacterial accumulation may only increase mortality when the immune system deteriorates with age.

In summary, bacterial accumulation early in adulthood seems to be controlled by gut immunity and with aging there is a decline in immune response and a deregulation in controlling bacteria proliferation which is strongly and inversely correlated with longevity[25].

5. *C. elegans'* longevity is influenced by multiple factors

Along with the effect of bacteria accumulation, bacteria can affect host longevity by other mechanisms. First, bacteria are the food source for *C. elegans*, therefore, nutritional quality and bacterial metabolites may influence host aging[33]. Moreover, dietary restriction has been reported to extend the lifespan of worms, flies and mice[34]. Second, bacteria may cause pathogenic infections by creating a persistent infection or by producing toxins[33,35].

5.1 Pathogenic bacteria

C. elegans possess a grinder that destroys all bacterial cells[15,22], however, pathogenic bacteria are able to pass through the grinder intact, even in young *C. elegans*. Once in the intestine bacteria are capable of proliferating and killing the nematode[36]. Bacteria such as *Pseudomonas aeruginosa*[37], *Serratia marcescens*[38,39], *Salmonella enterica* and *Enterococcus faecalis* are known pathogenic bacteria for *C. elegans*[35].

S. marcescens is a Gram-negative enterobacteriaceae extracellular pathogen that causes disease in plants and in a wide range of both invertebrates and vertebrates hosts[38]. In humans, it is an opportunistic pathogen associated with hospital infections and nosocomial infections[18]. Given its pathogenic proprieties, this bacteria were widely adopted as a model to study the genetic basis of virulence[6,17].

S. marcescens is also a pathogen for *C. elegans*, however it is unknown if the two species co-exist in nature, even though this is strongly suggested by the fact that both are common in soils[6]. *S. marcescens* is capable of establishing a persistent intestinal infection that kills worms in 6-9 days[18,39]. In the first 6 hours after infection it is already possible to find bacteria within the intestinal lumen and after 24 hours, a clear distension of the intestinal lumen is visible. 48 hours after infection, there is a strong drop in the rate of egg laying and worms start do die after 72 hours of contact[39].

In addition to bacterial interactions, *C. elegans* longevity is also influenced by the rate of mitochondrial respiration and by the insulin/IGF signaling pathway – a "master key" in immune response and longevity regulation[34].

6. *C. elegans'* longevity and the insulin/IGF signaling pathway

C. elegans possesses a conserved innate immune system that is pathogen-specific[40,19] and decline as worms age[25,41]. One extremely important signaling pathway in the insulin/IGF signaling pathway (IIS pathway).

The IIS pathway is one of the most studied and well-characterized pathways in *C. elegans* (Figure 4), since it is an important stress-resistance pathway with implications in processes such as dauer formation[16,42], stress-resistance and longevity[43]. In general, the pathway consists of the receptor tyrosine kinase DAF-2 (an insulin/IGF-1-like receptor) that controls the fork-head-family transcription factor DAF-16 (the sole orthologue of FOXO transcription factor) [44]. When the receptor DAF-2 is activated, it initiates a kinase cascade that results in phosphorylation and repression of DAF-16. DAF-16 phosphorylation prevents it from entering the nucleus where it is necessary for the transcription of numerous genes related with innate immunity and stress response[19,22,30,45] (Figure 4).

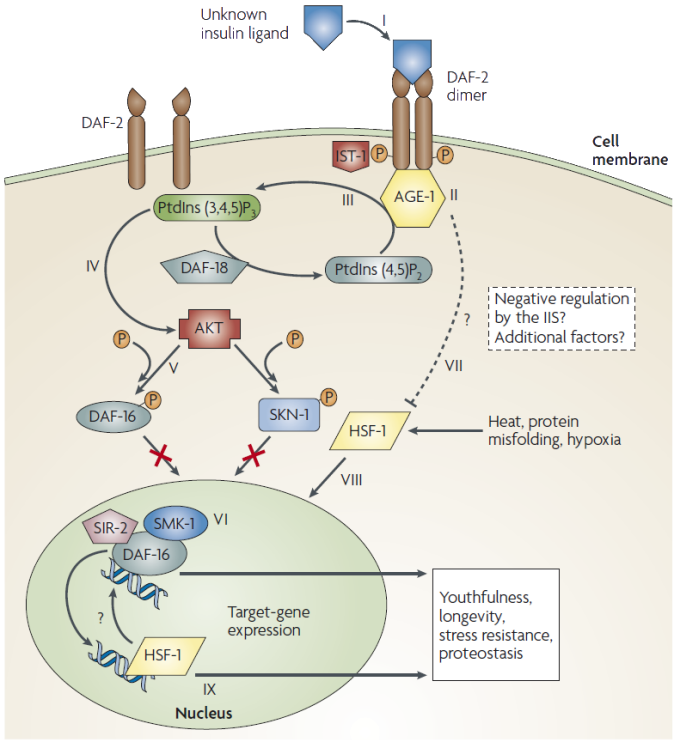

Figure 4 – Insulin/IGF signaling pathway

The pathway is activated with the DAF-2 receptor that maintains DAF-16 inactivated in the cytoplasm. In the absence of DAF-2, DAF-16 enters the nucleus where activates genes related with immune response and stress resistance. HSF-1 is also a key player that regulates gene expression longevity and stress resistance genes.

From Choen and Dillin 2008

daf-2 mutants have an increased resistance to heat, hypoxia, heavy metals and bacterial pathogens[42]. This increased resistance requires the activation of DAF-16, which in the absence of DAF-2 is capable of entering the nucleus[42]. Previous studies demonstrated that activation of DAF-16 triggers the expression of antimicrobial genes, such as the lysozyme lys-7[19]. This gene is upregulated when *C. elegans* is exposed to *Serratia marcescens*.

The lifespan of *C. elegans* is also regulated by the IIS pathway. Mutations in *daf-2* gene are related with an increased lifespan, whereas mutations in *daf-16* shorten lifespan. In that way, the activation of DAF-16 is necessary to activate the expression of genes related with aging[22].

Long-lived *daf-2* mutants also have lower levels of bacterial accumulation in their gut, however it is not clear whether this is a cause or an effect of *daf-2* longevity[25,31]. Mutations that diminish insulin-like signaling also increase lifespan and stress resistance in *Drosophila*[46] and mice[47], suggesting that the effect of this pathway on longevity is conserved.

In *C. elegans*, the increase in lifespan related with the IIS pathway has been associated with its expression in the intestine. Studies show that expressing *daf-16* in the intestine increases lifespan by 50-60%, which seems to indicate that the intestine is an important organ in IIS-mediated lifespan extension[43].

In summary, the IIS pathway involves increasing lifespan by regulating the entry of transcription factors such as DAF-16 into the nucleus. Another transcription factor that is involved in this pathway is the heat-shock factor-1 (HSF-1). In normal conditions, DAF-2 is activated and keeps DAF-16 and (probably) HSF-1 inactivated in the cytoplasm[48] (Figure 4).

6.1 The Heat-Shock Factor-1

In vertebrates there are four major heat-shock factors (HSF), while in *C. elegans* only one HSF homolog exists – HSF-1[49]. HSF-1 is the key regulator of the cellular and organismal response to heat stress and is conserved in all eukaryotes[50]. It is a leucine-zipper-containing transcription factor that controls the expression of small heat-shock proteins (HSP). One example is the HSP-16 family, which are molecular chaperones that prevent protein and cellular damage following stress[48,51]. In basal conditions, HSF-1 exists as a monomer in the cytoplasm and nucleus, whereas in stress conditions it becomes a trimer that accumulates in the nucleus where it binds to heat shock elements in the promoter region of HSP genes[52].

Alongside its stress response function, HSF-1 also contributes to processes such as development, growth, aging, immunity, reproduction and has been implicated in protein miss-folding diseases, such as Huntington's and Alzheimer's diseases[49]. In *C. elegans*, mutations in the *hsf-1* gene affect heat-shock response, larval development, egg-laying behavior and longevity[53].

In recent years, HSF-1 has been directly implicated in lifespan regulation. In *C. elegans*, inhibition of HSF-1 leads to decreased lifespan, since *hsf-1* mutants live, on average, 7 days less comparing with wild-type animals[30,53]. Conversely, overexpression of *hsf-1* is related to lifespan extension[54].

Even though *hsf-1* expression is associated with longevity regulation, the exact mechanism by which HSF-1 influences lifespan is not totally clear. Several studies have linked HSF-1 function with the IIS pathway, since the role of HSF-1 seems to be correlated with DAF-16[54].

One evidence is the fact that *daf-16* is required for *hsf-1* overexpression, although both genes operate independently from one another[54]. Additionally, it seems DAF-16 and HSF-1 increase longevity, at least in part, by increasing small heat-shock protein (sHSP) levels which prevent aggregations of unfolded proteins[54].

Another mechanism by which HSF-1 could increase lifespan is correlated with collagen regulation[52]. Collagen has been shown to have cytoprotective proprieties that are related with longevity. In a recent study, authors show that multiple genes involved in cuticle structure, including collagen genes, are enriched upon HSF-1 activation[52].

7. Selection of gut microbiota

7.1 Indirect selection

Numerous studies have proposed that manipulation of gut microbiota can increase host lifespan, suggesting a possible evolutionary-based strategy to extend longevity[21]. In a recent study, interaction between a host and its gut microbiota was analyzed from a new angle, where the performance of the hosts is improved by altering their microbiota[55]. Artificial selection can be applied on the gut microbiota in an indirect manner. The idea is to select gut microbiota by choosing a host trait that is directly influenced by its microbiota. The host trait should be easily measured and must have a strong correlation with the microbiota and with host fitness[55]. Such a trait can be, for example, the longevity of the host, since the microbiota have a large impact on host longevity.

7.2 Multilevel selection

Selection can act at multiple biological levels[56] and this interaction between different levels can be very important for microorganism evolution, mostly pathogenic ones. Multilevel selection is often studied in a context of antagonistic relationships[56] which can be interpreted as a trade-off between virulence and transmission[57].

Theories of virulence suggest that pathogens should evolve to a less-virulent state, since harming the host would be detrimental to a long-term survival strategy[57]. However, competition between pathogens should favor the one with higher proliferative and invasive rates, which translates in higher virulence levels[57]. In that way, within an infected host, individual fitness is central, while at the population level, efficient transmission between hosts is the critical component[58].

This kind of paradox can be overcome with a theoretical multilevel selection perspective, where traits that are costly at lower levels (individual level) can be beneficial at higher levels (host level) [59,60]. Virus evolution is one of the most studied examples, with selection occurring both within infected hosts and between hosts via transmission. Within the host, individual selection is prevalent since a fast-replication virus will outcompete a slower strain. However, if rapid viral replication incapacitates the host, the fast-replicating virus may not be transmitted as frequently as the slower strain, meaning that at a higher level, selection favors the less virulent strain[56,61].

In summary, high virulence should have a higher benefit at lower levels, due to individual competition, while low virulence should only be selected at higher levels of selection, where individual selection is minimized.

Nonetheless, multilevel selection theory still has some conceptual problems, namely, there is an ambiguity regarding the definition of a "higher level trait", as well as a lack of a precise definition of "higher level fitness" [62].

8. Main questions

The main goal of this work was to investigate if gut microbiota evolution is directly related with changes in host traits, namely in host longevity. To do this, *C. elegans* was used as a model host and *S. marcescens* as gut microbiota. A second major objective was to explore the role of different levels of selection in pathogenic bacteria evolution. In order to fulfil these goals, we specifically aimed to:

1. Characterize the survival rate of wild-type N2 and *hsf-1* mutant when feeding on different bacteria, mainly pathogenic bacteria;
2. Create experimental conditions favoring high or low levels of individual selection;
3. Correlate different levels of selection with pathogenic bacteria evolution;
4. Select gut microbiota able to colonize *C. elegans'* gut and alter its longevity;
5. Prove that the microbiota can be indirectly selected;
6. Validate the effect of evolved gut microbiota on *C. elegans'* longevity.

II. Material and Methods

1. Strains and maintenance

In this work, two different strains of *Caenorhabditis elegans* were used: the N2 Bristol strain and the PS3551 strain, which has a mutation in the *hsf-1* gene. Both were obtained from the Caenorhabditis Genetics Center – CGC. Populations were maintained, according to standard conditions[63,64], at 20°C in nematode growth medium (NGM) plated with *Escherichia coli* OP50.

Synchronized populations were obtained by treating adults with a bleach solution which allows unhatched embryos isolation. Embryos stayed in M9 buffer over-night with shaking, so as to obtain a synchronized population of L1 individuals, that arrested their development due to absence of food[65,66]. Hatched L1 were then seeded (transfer onto NGM plates) to start a new generation.

In order to avoid contaminations, some alterations were applied to the standard protocol, namely M9 buffer was supplemented with gentamicin (10µg/ml). Additionally, all plates with maintenance worms had NGM supplemented with kanamycin (50µg/ml) and every two generations supplemented with ampicillin (100 µg/ml).

Two different strains of *E. coli* OP50 were used: one with mCherry fluorescence (red) associated with kanamycin resistance and another with GFP fluorescence (green) associated with ampicillin resistance. Both inserts are chromosomic. The use of these strains allows the alternation of antibiotics, as well as easy checking for carrying-over bacteria.

Worms used for experiments were seeded as unhatched embryos (right after bleach treatment), given that development arrest could influence survival of individuals[66]. 4th stage larvae - L4 (approximately 48 hours after seed) worms were used in all experiments. Different bacteria were used during the course of our experiments:

- *E. coli* OP50, both with and without fluorescence;
- *E. coli* MG1655;
- *E. coli* IAI1 with mCherry fluorescence (plasmid with ampicillin resistance) and without fluorescence;
- *E. coli aroD*;
- *Serratia marcescens* db10 (tetracycline resistant) and db11 strain (tetracycline, kanamycin and streptomycin resistant).

All bacteria came from the CGC and before seeding (bacteria plating), bacteria were grown overnight at 37°C with shaking in liquid lysogeny broth medium (LB) supplemented with antibiotics. Bacteria were stored at 4°C in LB plates supplemented with their respective antibiotic and every two weeks transferred to a new plate.

2. Survival assays

Survival assays started with L4 individuals being transferred onto plates, effectively making L4 stage 'day 0'. In order to avoid starvation and overcrowding by progeny, worms were transferred daily to new plates during the fertile phase (approximately 6-7 days). Later, individuals were transferred every second day. An individual was considered dead if it failed to either move or respond to touch and did not show any sign of pharyngeal pumping. Worms that show an egg laying defect (bagging) or died from vulva bursting were considered as dead individuals. Worms that disappeared, probably due to crawling off the plate or disintegration after dying, were treated as censored data.

2.1 Transferring C. elegans with filters

In this work, a new method (inspired on the work of K. Lew and J. Miwa[67]) of transferring *C. elegans* was developed, which allows the transfer of a large number of individuals and at the same time separate adults from eggs, L1 and L2 larvae.

In this protocol, a 40µm filter was used to separate and transfer approximately 300 adults at once (see S1.2 for more details). Plates were washed with M9 buffer and *C. elegans* were filtered and washed two times with M9. Adults did not pass through the filter and were recovered with a glass pipet and transferred in buffer drops to a new petri dish.

To test the efficiency of this new protocol, a survival assay with wild-type N2 and *hsf-1* mutants was performed, where individuals were passed daily onto new plates seeded with *E. coli* OP50 by filtering. With this protocol, it was possible to perform and recover a standard survival curve (Figure S3), were, as expected, N2 individuals had higher survival (*hazard ratio=0.515*) compared to *hsf-1* mutants (*p-value<0.0001*). Otherwise mentioned, his method was used in all experiments.

2.2 Survival in Peptone-Free NGM

During the experimental evolution protocol, peptone-free NGM (PFN) plates were used in order to guarantee that only *S. marcescens* from the gut was selected, since this medium was effective in preventing bacterial growth[68]. Given this, survival of N2 individuals fed on *S. marcescens* db10, db11 and *E. coli* OP50 mCherry (used as control) was analyzed. All plates were 90mm PFN plates seeded with a specific strain of bacteria, which were replicated three times. Before the seed, bacteria grew in an over-night liquid culture with shaking and was concentrated until an OD_{600}=10 in order to form a lawn. Otherwise mentioned, this medium was used in all following experiments.

2.3 Statistical analysis

A Cox Proportional Hazards Regression survival analysis was performed using the *coxme* function in R, version 3.3.1. This function fits a Cox model containing mixed effects, allowing the use of fixed (e.g. Structured condition) and random effects (e.g. Replicates). A *p-value<0.05* was considered to indicate significance of effects. For more details see S7.

3. Gut colonization

During the survival experiment with pathogenic bacteria (S1.3), the ability of those bacteria to colonize *C. elegans'* gut was analyzed. Every day, two individuals from each condition were selected at random and crushed in order to recover gut bacteria. Bacteria recovery was done by crushing individuals in 5µl of PBS. Different dilutions were plated in 10µl drops in LB and minimum medium with and without uracil, thus distinguishing between experimental bacteria and *E. coli* OP50 mCherry. As positive control, *E. coli* MG1655 was used (capable of growing in minimum medium with and without uracil) and as negative control, *E. coli* OP50 mCherry that only grows in minimum medium with uracil.

4. Mutagenesis

In order to maximize genetic diversity in our bacterial populations a chemical mutagen - ethyl methanesulfonate (EMS) – was used. The protocol was followed according to Parkhomchuk *et al.*, 2009 during different time-points (15, 30, 45, 60 minutes). Control population was subject to the same protocol, however, no EMS was added. *E. coli* OP50 and *S. marcescens* db10 were used in this protocol.

Mortality rates of bacteria were evaluated by comparing CFUs of control and mutated bacteria (S2). The 15-minute mutated population of *S. marcescens* was used in the experimental evolution.

5. Levels of selection

In this work, one objective was to develop experimental conditions to create different levels of selection. For that, in association with the Techinico-scientific Support (TSS) team at the Instituto Gulbenkian de Ciência (IGC), we developed a honeycomb-like 3D scaffold composed by 594 individual cells. This scaffold restricts the movement of individuals by confining then inside a single cell and, ideally each worm has access to a limited number of different bacterial colonies.

Bacteria were placed in 150mm plates in order to obtain approximately 500 individual bacterial colonies per plate. In the **structured condition**, *C. elegans* were placed in the petri dish and after 20 minutes the 3D scaffold was added. In contrast, no scaffold was added in the **non-structured condition**, allowing worms to move freely. The scaffold stayed on over-night and was removed the next morning.

6. Experimental Evolution

For the experimental evolution protocol, N2 individuals were exposed to wild-type and mutant *S. marcescens* db10 populations in structured and non-structured conditions. Each treatment was replicated three times, totaling 24 evolving populations. Individuals were selected in three different time-points and their gut bacteria was recovered in order to start a new round of selection. After one round of selection, survival of individuals exposed to derived bacteria selected at the different conditions was analyzed in the non-structured condition, in order to only test the impact of the initial environment.

6.1 Experimental protocol

Approximately 200 L4 individuals were transferred in M9 buffer drops, onto 150mm NGM-tetracycline plates. These plates had been previously seeded with wild-type or mutant *S. marcescens* and the 3D scaffold was added in the structured condition plates.

The following day, the 3D scaffold was removed and all plates were washed with M9 buffer. Individuals were transferred onto 90mm PFN plates seeded with *E. coli* OP50 mCherry, where they were maintained (Figure 5). Dead individuals were removed daily in order to guarantee that only individuals which die at specific time-points were considered.

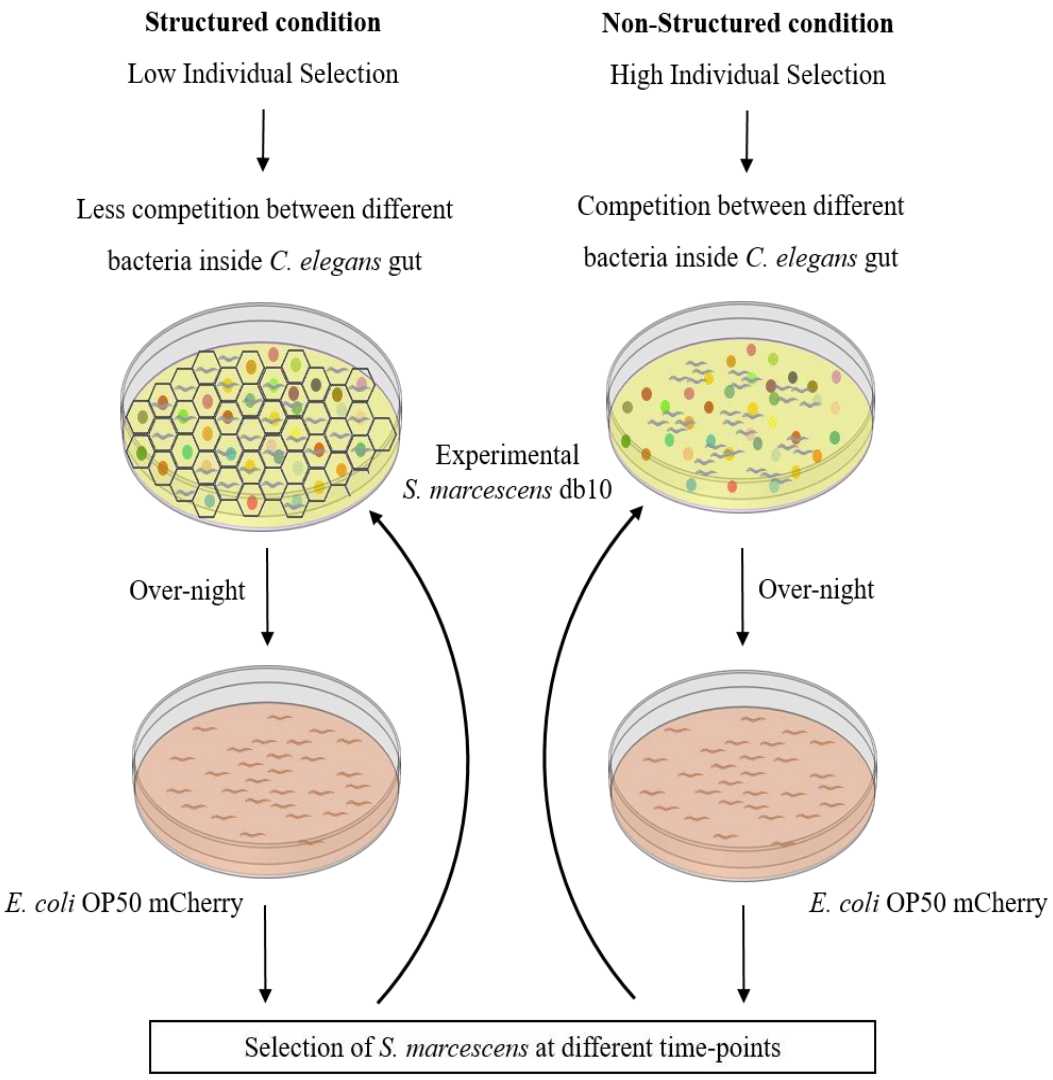

Figure 5 – Scheme of experimental evolution protocol.

6.2 Selection time-points

Three different selection time-points - T1, T2 and T3 – were defined in order to select bacteria at different stages of *C. elegans'* lifespan. T1 was defined as the time when 5% of the total population was dead. T2, which controls for random selection, was defined as the mid-point of worms' lifespan where dead individuals corresponding to 5% of total population were selected. T3 individuals were the final 10% that stayed alive.

6.3 Selection of individuals

At each time-point, gut bacteria of selected individuals were recovered. Individuals were selected using 5µl droplets of PBS and transferred to an individual well in a 96 well plate. Worms were crushed with a 0,5µm pestle and the solution was diluted in PBS and plated in 5µl drops in both LB and LB-tetracycline. To control for contaminations, a random sample was selected to be plated in both LB-kanamycin and LB-ampicillin. Plates were incubated over-night at 37°C, and on the next day stored at 4°C.

6.4 Preparation for the next round of selection

Since T1 and T3 were separated by one week, a strategy to guarantee that all bacteria were approximately in the same stage had to be developed. Upon T3, samples from all time-points were individually transferred and plated once again in LB-tetracycline. That way, all samples had grown over-night at 37°C before being plated for the next round. 50 colonies from each sample were then selected and placed in liquid LB. OD_{600} for each sample was measured in order to guarantee that all samples had the same quantity of bacteria. These samples were seeded in 150mm NGM-tetracycline plates to be used in the following round of selection.

III. Results

1. *C. elegans* fed on different pathogenic bacteria have different lifespans

One objective of this work was to characterize *C. elegans'* survival when feeding on pathogenic bacteria. To do that, survival of wild-type N2 and *hsf-1* mutants fed on *S. marcescens* db10, *E. coli* IAI1 and *E. coli* IAI mCherry was analyzed (S1.3).

There were no differences in survival rates of N2 individuals (Figure 6A) feeding on *E. coli* IAI1 or *S. marcescens* (*p-value=0.304*). Lifespan of individuals fed on *E. coli* IAI1 or *E. coli* IAI1 mCherry was different (*p-value<0.0001*), being that worms fed on *E. coli* IAI1 mCherry had a lower probability of death (*hazard ratio=0.501*).

Unlike wild-type worms, *hsf-1* mutants (Figure 6B) had different survival rates when feeding on *E. coli* IAI1 or *S. marcescens* (*p-value=0.003*), being that *S. marcescens* reduced the risk of death (*hazard ratio=0.510*). There were also differences between survival rates of *hsf-1* worms when feeding on *E. coli* IAI1 or *E. coli* IAI1 mCherry (*p-value=0.0004*). However, unlike wild-type N2, *hsf-1* mutants survived less when feeding on *E. coli* IAI1 mCherry (*hazard ratio=1.608*).

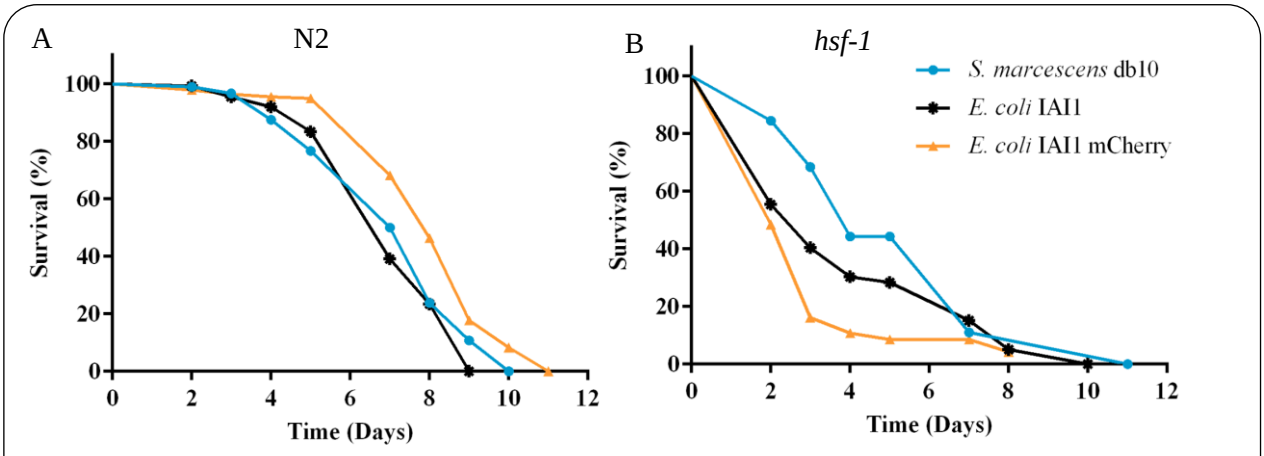

Figure 6 – Survival of *C. elegans* fed on different pathogenic bacteria.

A – wild-type N2 fed on *S. marcescens* db10 (*blue*) n=285; *E. coli* IAI1 (*black*) n=134; *E. coli* IAI1 mCherry (*orange*) n=229. **B** – *hsf-1* mutants fed on *S. marcescens* db10 (*blue*) n=45; *E. coli* IAI1 (*black*) n=130; *E. coli* IAI1 mCherry (*orange*) n=161. One replicate per condition.

2. *S. marcescens* is capable of colonizing *C. elegans'* gut

The main goal of this work was to evolve gut bacteria, thus it was necessary to verify if bacteria were indeed capable of colonizing *C. elegans'* gut. In the previous survival experiment, the ability of bacteria to colonize *C. elegans'* gut was analyzed by selecting every day two individuals from each condition and recover its gut bacteria. Note that individuals were feeding on *E. coli* OP50 mCherry, which is distinguishable from non-fluorescence *E. coli* IAI1 and *S. marcescens* colonies.

E. coli IAI1 mCherry lost fluorescence easily, since an over-night growth was enough for non-fluorescence colonies to appear (data not shown). Thus, this strain was excluded from following experiments.

Concerning the two other bacteria, *S. marcescens* and *E. coli* IAI1 were found in the gut 24 hours after infection, since both bacteria grew in LB (Figure 7a, d) and in minimum medium plates (Figure 7b, c, e, f). Interestingly, results show that individuals fed on *S. marcescens* did not have *E. coli* OP50 mCherry in their gut (all colonies were not fluorescent) (Figure 7a-c), while individuals fed on *E. coli* IAI1 had both *E. coli* strains in their gut – there were both fluorescent and non-fluorescent colonies (Figure 7g-i, n-p). Moreover, non-fluorescent colonies from individuals fed on *S. marcescens* or *E. coli* IAI1 in both LB and minimum medium were morphologically different (Figure 7a-f, j-m) – *S. marcescens* colonies were more compact and less translucent.

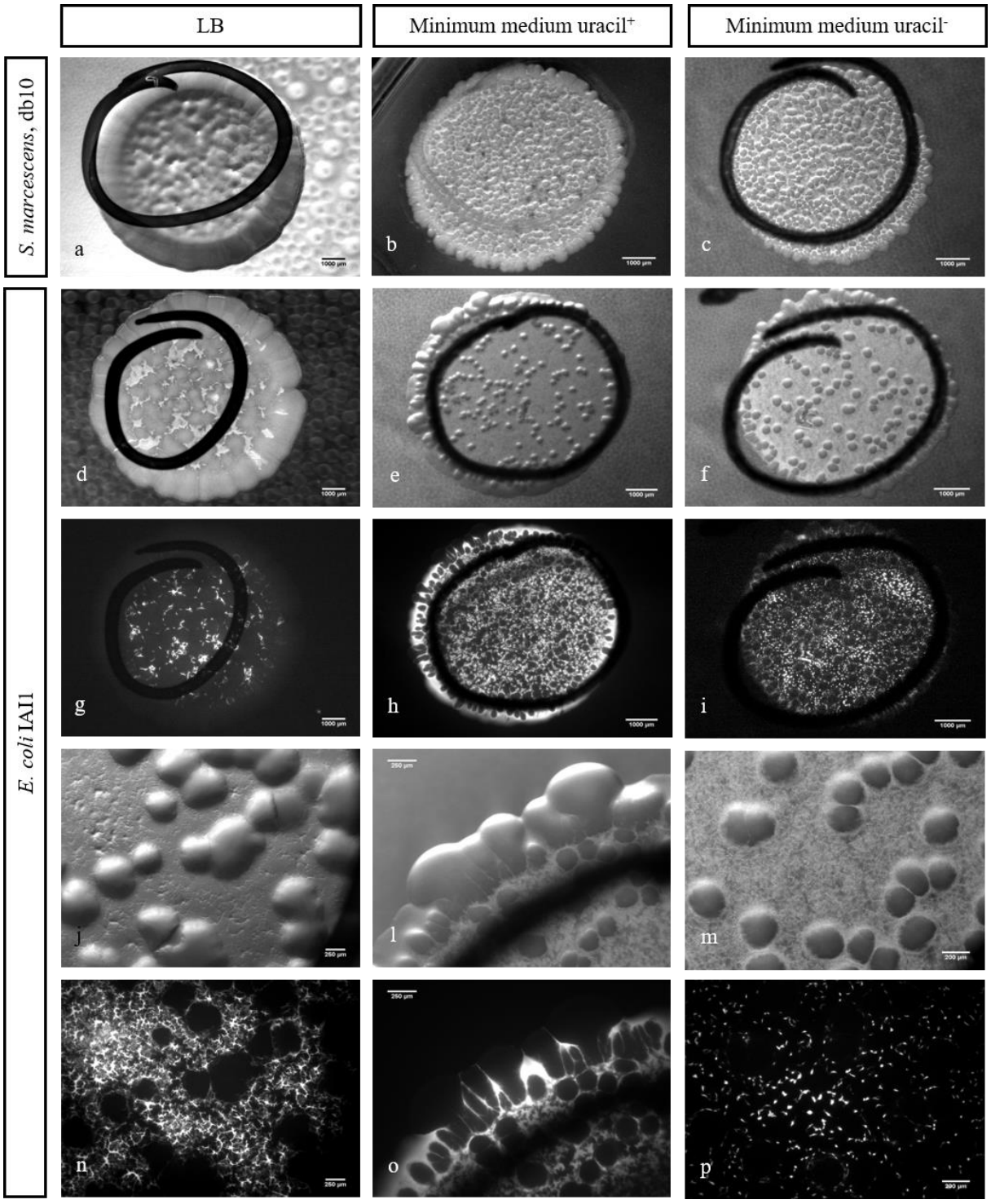

Figure 7 – Gut bacteria recovered from *C. elegans* fed on *S. marcescens* and *E. coli* IAI1.

First column – LB medium; **second column** – minimum medium with uracil; **third column** – minimum medium without uracil. **a-f, j-m:** brightfield stereoscope images; **g-i, n-p:** mCherry fluorescence stereoscope images. Scale bar=1mm (**a-i**), 250µm (**j-l, n-o)**, 200µm (**m,p**). Marker lines encircle initial plating area.

On day 6 of the experiment, *S. marcescens* and *E. coli* IAI1 could still be found in the gut. However, on day 7, a morphological change in non-fluorescent colonies derived from individuals fed on *E. coli* IAI1 was visible – colonies became very similar to *S. marcescens* non-fluorescent ones (Figure 8).

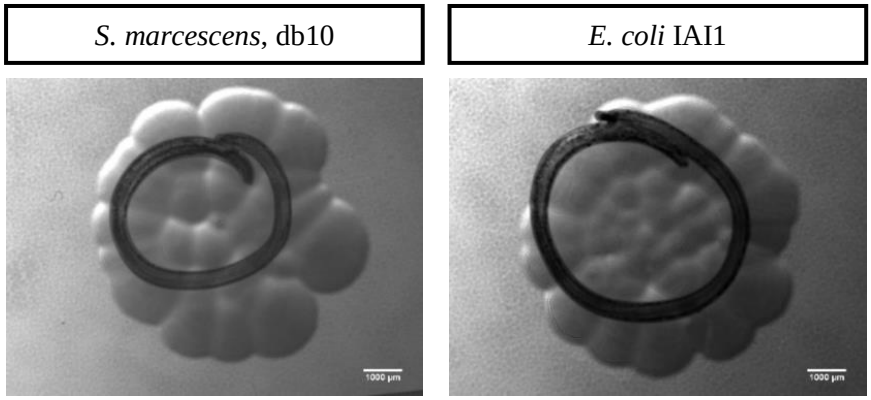

Figure 8 – Bacteria recovered on day 7 from individuals fed on *S. marcescens* and *E. coli* IAI1.
Brightfield stereoscope images, scale bar=1mm. Marker lines encircle initial plating area.

To understand this result, a portion of each bacterial colony was recovered and streaked in LB and LB-tetracycline plates. Results show *E. coli* IAI1 plates were contaminated with *S. marcescens* (data not shown), consequently it was not possible to conclude if *E. coli* IAI1 is indeed capable of colonizing *C. elegans'* gut. Moreover, the survival of individuals fed on *E. coli* IAI1 (Figure 6) could be affected by the presence of *S. marcescens*.

Given these results, *S. marcescens* was chosen as experimental bacteria, since it is capable of colonizing *C. elegans'* gut.

3. Experimental Evolution

In the experimental evolution assays, wild-type N2 and *hsf-1* mutant individuals were exposed to a mutant population of *S. marcescens* db10 in NGM plates with and without structure (S4). During this experiment, survival of individuals was not analyzed and selection time-points were calculated based on a standard curve previously obtained with *S. marcescens* (Figure 6).

Thus, the number of individuals to be selected in each time-point represent a fix percentage (5 or 10%) of the initial population size. Given this, in each time-point there should be a predictable number of individuals to be selected – selectable *C. elegans*. However, during four rounds of selection, the frequency of selectable individuals in each time-point was not as predicted. Results from N2 individuals (Figure 9A) did not present any clear pattern, while *hsf-1* mutants showed an increment in the percentage of dead individuals at T1 (Figure 9B). Since increased virulence was expected to be selected in T1 (which is in agreement with obtained data), a survival assay for *hsf-1* mutants exposed to ancestral and derived *S. marcescens* was performed (S1.4) in order to test if this pattern reflected a possible effect of selection. However, no differences in survival were found (Figure S4).

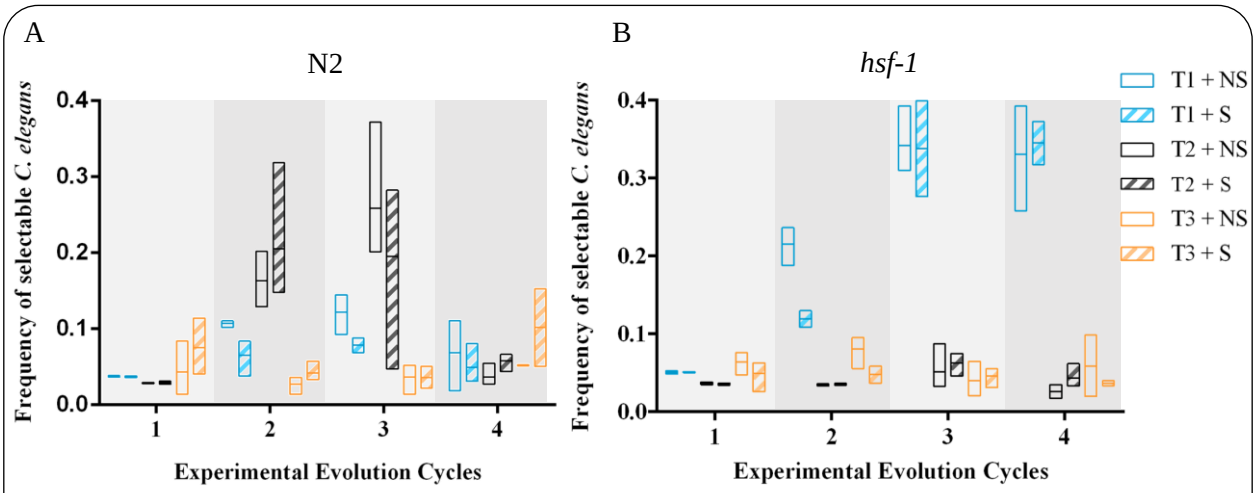

Figure 9 – Frequency of selectable *C. elegans* for each time-point.

Y axis represents the percentage of individuals that could be selected in each time-point. **A** – wild-type N2; **B** – *hsf-1* mutants. Non-structure (hollow bars); Structure (striped bars); T1 (*blue*); T2 (*black*); T3 (*orange*); three replicates per condition. Horizontal line represents mean value.

At the end of the fourth round of selection, a possible cross contamination between all experimental populations was found (data not shown), which indicates that all populations were indistinguishable. Consequently, the experiment was abandoned and a new cycle of evolution was started.

In this new cycle of experimental evolution, only N2 individuals were used and exposed to a new mutant population of *S. marcescens*. Peptone-free NGM (PFN) plates seeded with *E. coli* OP50 mCherry were used in order to guarantee that only gut *S. marcescens* was selected. As before, the structured and non-structured conditions were used and individuals were selected at the three different time-points to recover their gut bacteria.

3.1 Higher genetic variability causes lifespan differences upon decreased individual selection

Before the beginning of this new cycle, the impact of bacterial genetic variability in *C. elegans'* survival was analyzed by looking at how survival of N2 individuals was different while feeding on wild-type and mutant *S. marcescens* with and without structure.

There was no difference in survival between individuals fed on wild-type or mutant bacteria (*p-value=0.32*) (Figure 10). However, when the structured and non-structured condition was taken into consideration, a difference in *C. elegans* survival was detected. Individuals fed on mutant bacteria had lower probability of death when in the structured condition (*hazard ratio=0.716* and *p-value=0.025*). On the contrary, worms fed on wild-type bacteria had no difference in survival in both conditions (*p-value=0.34*).

This result indicates that the two conditions were in fact different and had an impact on mutant bacterial competition, which was reflected in *C. elegans'* survival.

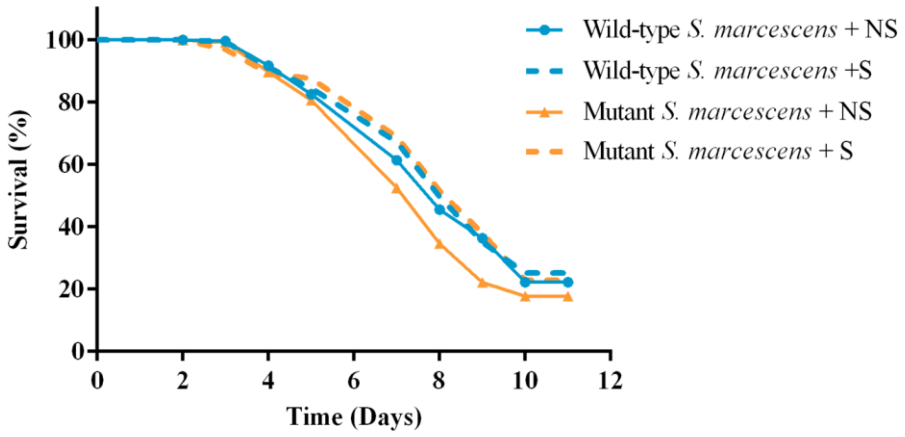

Figure 10 – Survival of N2 individuals fed on wild-type and mutant *S. marcescens*.
Wild-type and non-structure n=668 (*full blue*); wild-type and structure n=773 (*dashed blue*); mutant and non-structure n=591 (*full orange*); mutant and structure n=544 (*dashed orange*). Three replicates per condition.

3.2. One round of selection in mutant bacteria is sufficient to increase C. elegans' longevity

The main goal of this work was to test if it possible to select and evolve gut bacteria capable of altering *C. elegans'* longevity. Due to temporal constrains, only one round of selection was performed after which N2 individuals were exposed to bacteria previously selected at the different time-points and conditions. One way by which population evolution can be demonstrated is by comparing the effect of ancestral and derived bacteria on *C. elegans'* longevity. Ancestral and derived wild-type *S. marcescens* were not different (*p-value=0.29*) (Figure 10,11A) and there was no interaction with the structure (*p-value=1*). However, ancestral and derived mutant *S. marcescens* (Figure 10,11B) were different (*p-value<0.0001*) and *C. elegans* exposed to derived bacteria had a lower probability of death (*hazard ratio=0.734*). Still, the presence of structure had no impact on survival of worms fed on mutant *S. marcescens* (*p-value=1*).

This result shows that one cycle of selection was enough to cause differences in mutant *S. marcescens* populations, which translated in an increment of *C. elegans* longevity.

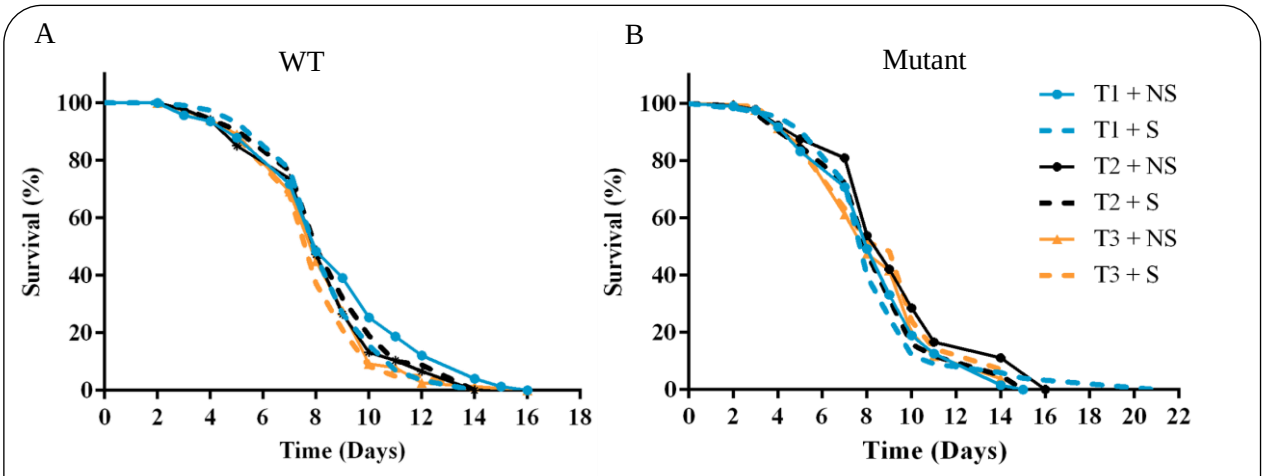

Figure 11 – Survival of N2 individuals fed on derived *S. marcescens*.

A – Wild-type bacteria; T1 non-structure n=620 (*full blue*); T1 structure n=685 (*dashed blue*); T2 non-structure n=862 (*full black*); T2 structure n=719 (*dashed black*); T3 non-structure n=729 (*full orange*); T3 structure n=706 (*dashed orange*). **B** – mutant bacteria; T1 non-structure n=1035 (*full blue*); T1 structure n=706 (*dashed blue*); T2 non-structure n=945 (*full black*); T2 structure n=794 (*dashed black*); T3 non-structure n=725 (*full orange*); T3 structure n=935 (*dashed orange*). Three replicates per condition.

Besides the comparison between ancestral and derived bacteria, population evolution could eventually be seen in different time-points. However, there was no effect on *C. elegans'* survival of selecting bacteria in different time-points for both wild-type and mutant *S. marcescens* (*p-value=0.073* and *0.313*, respectively) (Figure 11).

4. *C. elegans'* lifespan decreases when feeding on a mixture of bacteria

Since during experimental evolution PFN plates were used, it was necessary to analyze *C. elegans* survival in this new medium. Worms were exposed to *S. marcescens* db10, db11 and *E. coli* OP50 mCherry (S1.5).

As expected, there were no differences between survival rates of N2 individuals fed on either *S. marcescens* db10 or db11 (*p-value=0.63*) (Figure 12). However, individuals fed on *S. marcescens* db10 survived more (*hazard ratio=0.566*) when compared with worms fed on *E. coli* OP50 mCherry (*p-value=0.009*). The same happens with *S. marcescens* db11; worms fed on db11 had lower probability of death (*hazard ratio=0.484*) comparing to *E. coli* OP50 mCherry (*p-value=0.001*), which was surprising.

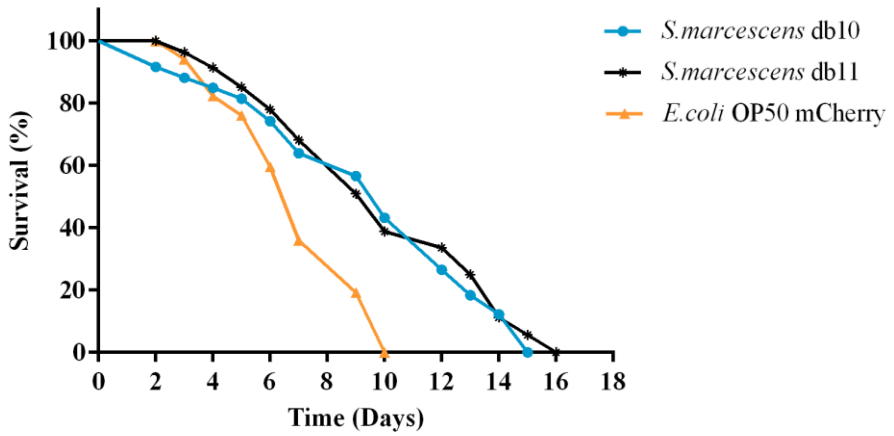

Figure 12 – Survival of *C. elegans* fed on different *S. marcescens* strains and *E. coli* OP50.
N2 individuals fed on *S. marcescens* db10 (*blue*) n=737; *S. marcescens* db11 (*black*) n=657; *E. coli* OP50 mCherry (*orange*) n=525; three replicates per condition.

Given this unexpected result, individuals from *E. coli* OP50 plates were picked and their gut bacteria recovered to confirm whether worms were feeding on *E. coli*. Results indicated a contamination with *S. marcescens* db11 (data not shown) giving rise to a new hypothesis where the interaction (most likely competition) between *S. marcescens* db11 and *E. coli* OP50 could be the cause of lifespan decrease[84].

To test this hypothesis, a second experiment was performed where the survival of N2 individuals fed on a mixture of bacteria, was analyzed. Animals were exposed to a mixture of *S. marcescens* db10 and db11 and then transferred to *E. coli* OP50 mCherry.

Since the purpose of this experiment was to test the role of bacterial competition in *C. elegans'* survival, the experiment was performed in structured and non-structured conditions, which provide different opportunities for bacterial competition to occur.

To confirm initial bacterial proportions, the mixture was plated in LB-tetracycline and LB-tetracycline-streptomycin. *S. marcescens* db10 were at higher proportion in initial plates – 98%, which could be explained by a possible higher growth efficiency or simply by the fact that the mixture was not balanced (OD_{600} results are not an exact measurement).

Once more, *C. elegans* fed on a mixture of bacteria (Figure 13) had higher probability of death than individuals fed on single (Figure 12) *S. marcescens* db10 (*hazard ratio=0.346*) or db11 (*hazard ratio=0.300*) (*p-value<0.0001*, in both cases). Accordingly, no difference was found when comparing N2 worms fed on either the mixture or on *E. coli* OP50 mCherry contaminated with *S. marcescens* db11 (*p-value=0.072*). Moreover, *C. elegans* in structured conditions had lower probability of death than individuals which could freely explore the environment (*p-value=0.002*).

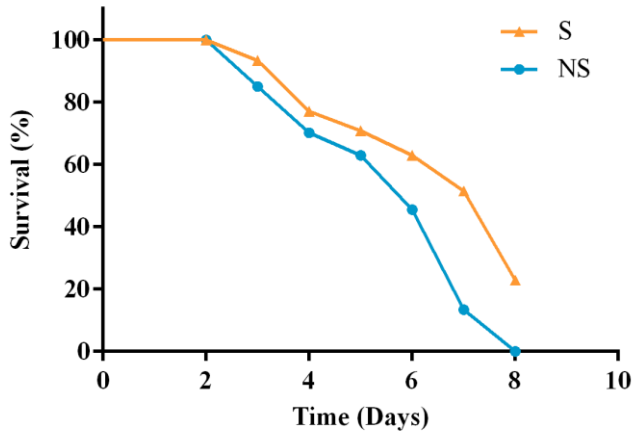

Figure 13 – Survival of *C. elegans* fed on a mixture of *S. marcescens* db10 and db11.
C. elegans N2 exposed to non-structure (*blue*) and structure (*orange*) conditions. 2 replicates per condition; Structure n=172; Non-Structure n=187.

4.1 C. elegans decrease in lifespan can be associated to S. marcescens db11 when in competition with E. coli OP50

Taking the previous results into consideration, it was interesting to test if the decrease in lifespan, and consequently higher virulence, was a general effect of bacteria competition or if it was a more specific one associated with one strain in particular.

One way to test this was to recover gut bacteria throughout time and analyze the proportion of different bacteria (S1.5, 6). This idea takes into account the assumption that more virulent bacteria should be in higher proportion in the first individuals to die, and over time it should be replaced by less virulent bacteria.

This effect should be stronger in the structured condition, where intra bacterial strain competition is expected to be lower giving an over-time advantage to the less virulent bacteria. If *S. marcescens* db10 and db11 had different levels of virulence due to competition with *E. coli* OP50, it should be possible to recover different proportions of the bacteria throughout time.

S. marcescens db10 started out in higher proportions inside *C. elegans'* gut and it frequency was even higher in the non-structured condition (*p-value<0.0001*) (Figure 14). Over time, there was a significant increase in *S. marcescens* db10 frequency (*p-value<0.0001*), which was especially pronounced in the structured condition (*p-value<0.0001*) (Figure 14).

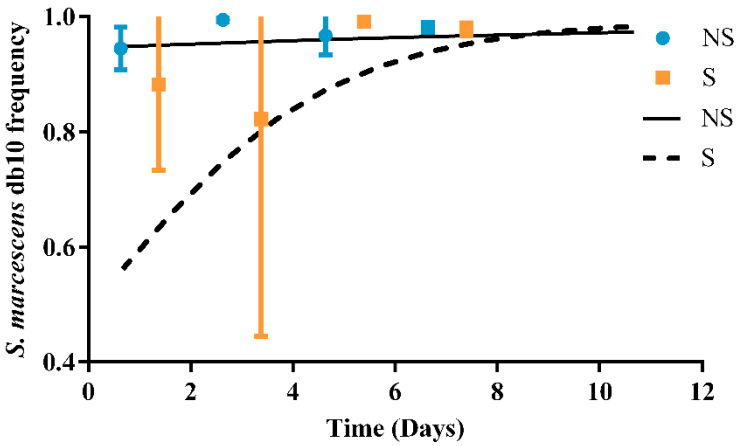

Figure 14 – Over time frequency of *S. marcescens* db10 inside *C. elegans'* gut.
Frequency of recovered bacteria at different time-points and conditions: Non-Structure (*blue*) and Structure (*orange*). Each point represents mean value for frequency calculated from 5 CFU measurements for *S. marcescens* db10 and db11; vertical bars represent standard deviation (SD). Black lines represent predicted model for non-structure (*full*) and structured (*dashed*) conditions (see S7).

5. Kanamycin resistance in *S. marcescens* is associated with trade-offs

During the initial experimental evolution protocol, NGM plates were supplemented with kanamycin in order to guarantee that only *S. marcescens* from the gut has selected. However, after four days, well-defined bacterial colonies without fluorescence could be seen on the plates (data not shown). This was an indicator that *S. marcescens* was growing on plates, since *C. elegans* were feeding on *E. coli* OP50 mCherry.

To confirm this result, *S. marcescens* ability to gain kanamycin resistance was tested (S5), given that antibiotic resistance could be extremely important for host-microbiota interactions. In hospital environments for example, antibiotic resistance is a growing concern that directly impacts the interactions with the host.

An over-night culture of *S. marcescens* was grown in LB, LB-tetracycline and LB supplemented with different kanamycin concentrations (12.5µg/ml, 25µg/ml and 50µg/ml). As expected, *S. marcescens* grew well in LB and LB-tetracycline. However, bacterial growth in the three different kanamycin concentrations was also visible and, as expected, bacteria grew more in lower antibiotic concentration (data not shown).

To confirm these results, the 25µg/ml liquid growth was re-grown in 25µg/ml and 50µg/ml kanamycin liquid culture and an increment in kanamycin resistance was found (data not show). In addition, the over-night growth in the three different kanamycin concentrations was plated in LB, LB-tetracycline and LB-kanamycin plates. *S. marcescens* from liquid culture was capable of growing in LB-kanamycin plates (data not shown) and the capacity of growing was proportional to initial kanamycin concentration in liquid medium (bacteria exposed to lower concentration grew less in plates).

However, bacterial growth in LB-tetracycline was less than expected (data not show), which seems to indicate a trade-off between the two antibiotic resistances. Once again, with the aim of confirming this results, a colony from LB-tetracycline plates was selected and plated it in LB, LB-tetracycline and LB-kanamycin. As a control, stock *S. marcescens* was also plated.

There were morphological differences between the different bacteria, manly in LB plates. Stock *S. marcescens* formed large bacterial colonies when grown in LB (Figure 15a), grown normally in LB-tetracycline (Figure 15b) and did not grow in LB-kanamycin (Figure 15c). Bacteria which were initially exposed to lower concentrations of kanamycin were not capable of growing in LB-kanamycin (Figure 15f) and grew large bacterial colonies in LB (same as control) (Figure 15d). However, bacteria which were initial exposed to 25µg/ml show morphological differences – large and small colonies (Figure 15g). These bacteria grow in LB-tetracycline and did not grow in LB-kanamycin (Figure 15h, i). Finally, bacteria that had been previous exposed to higher kanamycin concentrations only grow small colonies in LB (Figure 15j) and were capable of growing in LB-kanamycin and LB-tetracycline (Figure 15l, m).

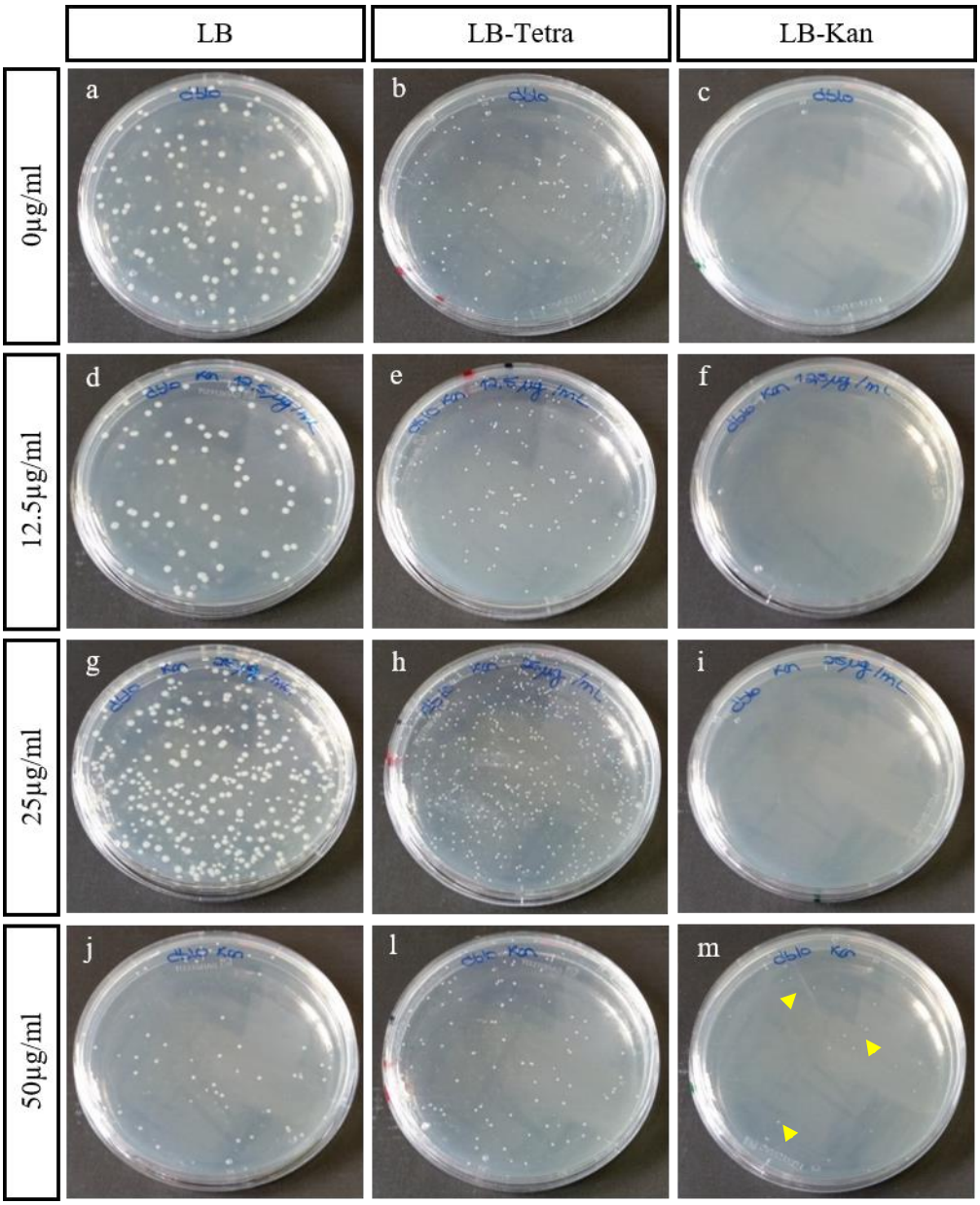

Figure 15 – *S. marcescens* from LB-tetracycline plates.

First column – LB**; second column –** LB-tetracycline**; third column –** LB-kanamycin**. First row –** stock *S. marcescens***; second row -** bacteria grew in liquid medium with 12,5µg/ml of kanamycin**; third row -** bacteria grew in liquid medium with 25µg/ml of kanamycin**; fourth row -** bacteria grew in liquid medium with 50µg/ml of kanamycin. Arrows indicate individual colonies.

In short, these results indicate that *S. marcescens* was capable of gaining kanamycin resistance in a very short period of time (over-night in liquid culture) and this resistance was associated with a trade-off in growth capacity (smaller colonies) and probably in tetracycline resistance. Given this, the NGM-kanamycin plates, could not be used as method to prevent S. marcescens growth on the plates.

5.1 S. marcescens kanamycin resistance has an impact on C. elegans' longevity

Given that a trade-off in growth capacity and kanamycin resistance was found in *S. marcescens* db10, it was relevant to test if this decrease in growth had an impact on *C. elegans'* longevity. To do this, survival of N2 individuals exposed to *S. marcescens* in NGM-kanamycin plates was analyzed. This experiment was performed together with, and in the same conditions as, the experimental evolution survival assay (Figure 10) so as to compare survival in both media (PFN and NGM-kanamycin).

There were differences in survival of individuals fed on *S. marcescens* in PFN or NGM-kanamycin plates (Figure 16). Individuals fed on wild-type bacteria survived less when in PFN plates (*hazard ratio=1.330* and *p-value=0.03*). The same happened with individuals fed on mutant bacteria – worms in PFN plates had a higher risk of death (*hazard ratio=1.761* and *p-value<0.0001*).

These results indicate that *S. marcescens'* kanamycin resistance increase *C. elegans'* longevity when comparing with survival of worms in PFN.

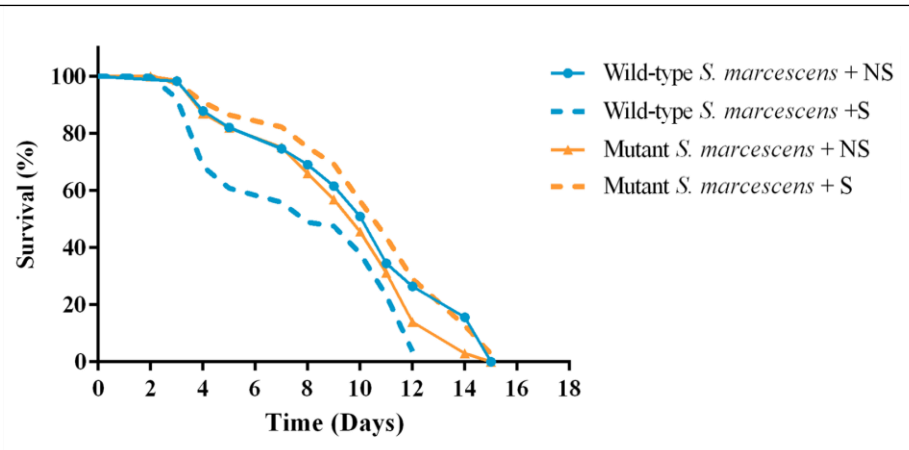

Figure 16 – Survival of N2 individuals fed on *S. marcescens* in NGM-kanamycin plates.
Wild-type and non-structure n=701 (*full blue*); wild-type and structure n=713 (*dashed blue*); mutant and non-structure n=723 (*full orange*); mutant and structure n=697 (*dashed orange*).

IV. Discussion

The main goal of this work was to investigate if gut microbiota evolution is directly related with changes in host longevity. As far as we know, until today no other work attempted to indirectly select microbiota by selecting a trait in an animal host[55]. Additionally, the role of different levels of selection in bacterial evolution was also explored, by creating experimental conditions where individual selection could be minimized or maximized.

To fulfill this goal, a high number of experimental populations as well as multiple experimental conditions were needed, which led to the development of the filtering protocol. Typically, in survival assays *C. elegans* are transferred from one plate to another by single picking process[25,31,63] which is extremely time-consuming since only one or two individuals are transferred at a time. Consequently, the number of worms and experimental conditions that could be applied in a relative short period of time is limited.

In order to overcome this problem, we developed a new and faster method of transferring adult *C. elegans* by using a filter instead of a picker. By implementing this protocol, the total number of experimental individuals could be greatly increased as well as the variety of experimental conditions. Another advantage is that worms have exactly the same experimental conditions that they have during maintenance, unlike other high-transfer-number protocols[71]. However, this protocol still requires some optimizations, namely *C. elegans* are transferred in liquid which remains on the plates for approximately 15-20 minutes and despite a much higher efficiency in separating adults from eggs and larvae, a very small number of larvae are not filtered and thus it is necessary to check plates for their presence.

1. *C. elegans* survival on different bacteria

One of the main objectives was to prove that diverse bacteria species and strains have different impacts on *C. elegans*' longevity. Survival curves of *hsf-1* mutants are not directly comparable to those in the literature, because individuals that died due to bagging or vulva bursting are usually excluded from analysis. In the present work, these individuals were taken into account, since evolved bacteria could have an impact on bagging[72].

As expected, wild-type N2 individuals fed on *S. marcescens* had the highest risk of death, followed by *E. coli* IAI1 (Figure S1A), which is in agreement with previous studies that describe both bacteria as pathogenic for *C. elegans*[23,39]. In contrast, *hsf-1* mutants, did not show differences in survival between worms fed on *S. marcescens* and *E. coli* IAI1 (Figure S1B). This can be explained by an initial high mortality rate due to egg laying defects which could overshadow the effects of the different pathogenic strains.

Another *E. coli* strain used to show that diverse strains have different impacts on *C. elegans*' longevity, was *E. coli aroD*. These bacteria are described as capable of increasing *C. elegans*' longevity[73], however, worms did not show an increment in longevity (Figure S1,S2). Though unexpected, these results can be explained, since the effect on longevity depends highly on the medium. The *aroD* gene encodes the enzyme 3-dehydroquinate dehydratase, a precursor to all aromatic compounds in bacterial cells and standard agar could have aromatic compounds that influence the effect on lifespan[73].

Keeping in mind the central aim of this study, it was necessary to re-test the survival of *C. elegans* when feeding on pathogenic bacteria[23,39,74].

Surprisingly, N2 individuals fed on *E. coli* IAI1 had higher survival than individuals fed on *E. coli* IAI1 mCherry (Figure 6A). This could be due to a possible cost of the plasmid conferring ampicillin resistance and mCherry fluorescence, which can be translated in lower proliferation rates. This hypothesis is consistent with bacteria easily losing the plasmid (see results 2) and with the fact that bacteria capacity to grow inside the gut is directly correlated to its pathogenicity[25,31].

In contrast to what was previously seen (Figure S1B), *hsf-1* mutants had different lifespans when feeding on different pathogenic bacteria, which points to a possible environmental effects. Surprisingly, individuals fed on *S. marcescens* had the highest survival rate (Figure 6B) and this kind of phenomenon could be explained by stress-response hormesis, which in this case is correlated with *hsf-1* mutation (wild-type N2 did not present signs of this phenomenon).

In the past years, hormesis has been extensively studied as a response to brief exposure to a stress that could result in up-regulation of stress induced genes which promote a defensive response[75]. This phenomenon has been associated with abiotic stresses, mostly heat-shock stress, and extension of *C. elegans'* lifespan. Most studies correlate the IIS pathway with hormetic stress-response to heat and lifespan extension[76]. This response depends on the activation of *daf-16* and *daf-18* genes[76]. *daf-16* mutants do not extend lifespan, but still present hormetic stress-response, while *daf-18* mutants do not extent lifespan and do not present increased stress response[77].

Leroy *et al.* (2012) was the first work to correlate hormetic response during *C. elegans* development with biotic stresses, namely pathogenic bacteria. Once again, low levels of IIS pathway activity were correlated with hormetic response and worms had higher levels of *hsp-16.2* gene expression, which is regulated by DAF-16 and HSF-1 transcription factors[78].

All previous works have shown a relationship between low levels of IIS pathway activity and increased stress response, which seems to contrast with the hypothesis that *hsf-1* mutants had an hormetic response to *S. marcescens*. However, despite a clear connection between heat stress and longevity, no study has ever shown the role of *hsf-1* gene in this response and its interaction with IIS pathway.

During the experimental evolution protocol, peptone-free NGM (PFN) [68] plates were used, thus it was important to test the impact of this new medium in N2 individual's survival. No difference was found between survival of individuals fed on *S. marcescens* db10 and db11(Figure 12), which is in agreement with previous reports[79,80].

Surprisingly *C. elegans* fed on *S. marcescens* in PFN plates survived 14-16 days (Figure 12), while individuals in NGM plates survived 9-10 days (Figure 6, S1, S2), which is in agreement with previous reports where *S. marcescens* kills *C. elegans* in approximately 6 to 9 days[38,39]. This difference in survival can be explained by an environmental effect of PFN that could be related with higher osmotic stress or absence of peptone. Peptone deprivation has been correlated with higher longevity via dietary restriction[68,81]. However, this effect is caused by a decrease in bacterial load[68], which is probably not the case in this experiment since plates were covered with a bacterial lawn. A second possible explanation is related with higher osmotic stress. However, higher salt concentrations are associated with a reduction in *C. elegans* survival[82,83]. Given this, it will be interesting to test what are the causes for increased lifespan in PFN and if this effect is visible when *C. elegans* feed on other bacteria.

During the same experiment, survival of individuals fed on *E. coli* OP50 contaminated with *S. marcescens* db11 was reduced (Figure 12), which points to a possible negative effect of bacteria competition in *C. elegans'* longevity. This hypothesis was tested in a second experiment confirming worm's survival was reduced when individuals were exposed to a mixture of *S. marcescens* db10 and db11(Figure 13).

There are two possible explanations by which bacteria competition could influence *C. elegans'* longevity. The first is related with different growth rates induced by bacteria competition and the second is related with bacterial metabolic profiles that can be changed during competition[84].

C. elegans immune response could also be important for this decrease in lifespan. During the worms' adulthood, the environment was mostly non-pathogenic bacteria, which could cause a decrease in *C. elegans* immune response[19,39]. To test this, *C. elegans* immune mutants, such as the *pmk-1* loss-of-function mutant[19], could be used in order to verify if the survival of individuals feeding on single bacteria or on a mixture is the same, in the absence of an immune response.

It is important to note that the experiment which was done does not allow us to see which bacteria are important for this competition effect and if this effect is general or if there is an environmental effect. A more detailed analysis was not possible due to time constrains, still, in order to overcome these gaps, the same experiment should be repeated with more replicates and with bacteria competing in pairs. Additionally, bacteria competition should be performed in plates and in *C. elegans* gut.

Nevertheless, a different protocol was used in order to test if the increment in virulence was specific for one bacterial strain. This protocol allows us to distinguish if two strains have differences in virulence due to bacterial competition since bacterial proportions should differ over time, if that is the case. However, if the bacterial proportion remains constant over time, it is not possible to distinguish whether virulence did not change or if it changed equally in both strains. Additionally, the presence of structure could give an over time advantage to the less virulent strain, since it will not be competing directly with a more virulent one.

S. marcescens db10 started at different frequencies in the structured and non-structured conditions (Figure 14), which was unexpected. This can be explained by the fact that in the structured condition the variance in frequencies between individuals was being increased. In the non-structured condition each individual had equal opportunity to feed on both *S. marcescens* db10 and db11, conversely, in the structured condition each individual had opportunity to feed on only one strain, increasing the variance between individuals. In fact, the structured condition had higher levels of variance between individuals compared to the non-structured condition (*p-value<0.0001*) (S7). One particular example was one individual from day 4 in the structured condition that had an extremely low frequency of *S. marcescens* db10 (TableS2). By removing the individual and running the statistical analysis again, the frequency of *S. marcescens* db10 on day 4 was the same in both conditions (Figure S5A). Moreover, by removing this individual, our predicted model shows that both bacteria should start in equal proportions (Figure S5B).

This difference in variance was not constant over time and was always associated with a higher frequency of *S. marcescens* db11. The fact that higher proportions of *S. marcescens* db11 only appeared in the first dead individuals seems to indicate that *S. marcescens* db11 increased its virulence when in competition, since a more virulent strain should be in higher proportion in the first dead individuals and over time it should be replaced by a less virulent one. However, since only one individual was found, we cannot be sure if this result is in fact a biological effect or a random effect of sampling. To confirm this, the same experiment should be repeated with more replicates.

In agreement with this, the frequency of *S. marcescens* db10 increased over time, mainly in the structured condition (Figure 14), which once again indicates that *S. marcescens* db10 had lower levels of virulence (comparing with *S. marcescens* db11) and that the structure condition prevents bacterial competition.

As a final remark, it is important to refer that *S. marcescens* db10 and db11 are genetically very similar and therefore, this effect is probably related with a specific mutation in *S. marcescens* db11. This effect could be related with the Type VI secretion system (T6SS) that has been describe as highly strain-specific and a major player in antagonistic bacterial competition, as well as in virulence[85,86].

2. Gut colonization

In this work, it was important to confirm that bacteria were capable of colonizing *C. elegans*' gut. One possible option was *E. coli* OP50 since it is a well-studied model organism and standard laboratory food for *C. elegans*[15,22]. Traditionally, *E. coli* OP50 is described as a non-pathogenic bacteria and therefore incapable of colonizing worms' gut[15,22]. Yet, recent works have report that *E. coli* OP50 is capable of gut colonization[25,31].

With these results in mind, *E. coli* OP50 mCherry was used as experimental bacteria. However, worms completely lost fluorescence inside their gut very quickly, which seems to indicate that *E. coli* OP50 mCherry is incapable of colonizing the gut. Note that mCherry fluorescence is chromosomal, so this result cannot be explained by loss of fluorescence.

In our work, gut colonization was considered as the capacity for bacteria to reach the intestine alive, proliferate and remain inside the gut, without an external source of the same bacteria. In contrast, studies claiming *E. coli* OP50 as being capable of gut colonization describe it as an increment in bacterial number as worms age[25] and as a capacity to proliferate in old animals[31]. Moreover, individuals were kept in the same bacteria during the entire experiment[25,31], thus is it not possible to say if bacteria remained inside the gut. Given our definition of gut colonization, *E. coli* OP50 mCherry could not be used.

Subsequently, *E. coli* IAI1 and *S. marcescens*, two known pathogenic bacteria for *C. elegans*, were tested since pathogenicity is related with gut colonization[23,38,39]. In fact, *S. marcescens* is described as a pathogenic bacteria able to produce a persistent infection[6,38,39]. Due to contamination problems (Figure 8), the data regarding *E. coli* IAI1 was lost. However, *S. marcescens* was indeed capable of colonizing the gut, since bacteria were found in the gut 7 days after infection.

3. Different levels of selection

One of the main objectives of this work was to develop experimental conditions where individual selection could be minimized or maximized. A first approach was to use different proportions of *C. elegans* feeding on bacteria (S3.1). However, with this protocol it was never possible to create a condition were individuals fed on only one bacterial colony (Figure S7, S8).

As an alternative and in association with the Techinico-scientific Support (TSS) team at IGC, we developed a honeycomb-like 3D scaffold that restricts individual's movements. With this scaffold, it was possible to create a condition where individuals were not able to explore their environment – structured condition. In this condition individual selection should be minimized since inside each worm gut there should be only one bacterial genotype.

In contrast, in the non-structured environment individuals could freely explore the environment and feed on different bacterial colonies, increasing individual bacterial competition within *C. elegans* gut.

Although results show that the two conditions are in fact different and impose different levels of selection, the protocol is not completely optimized. One particular example is the bacteria distribution on the plate. Ideally, each cell should have only one bacterial colony and results show cell without colonies and cells with a maximum of four bacterial colonies (Figure S9).

4. Experimental Evolution

In natural populations, the rate at which new mutations arise and their fitness effects are extremely important for evolution[87]. Typically, mutation accumulation experiments describe that the rate of single base mutations is of the order of $10^{-10} – 10^{-9}$ per base pair per replication, which is a relatively low mutation rate[88]. Since one aim of this work was to evolve gut bacteria, bacterial populations should have high levels of genetic diversity and for that, a mutagenic protocol was used.

It is expected that mutagenesis has a general negative impact on bacterial virulence, since most new mutations are neutral or deleterious, causing a decrease in virulence[87,88,89]. Taking into consideration a multilevel selection perspective, it is expected that low levels of virulence should be maintained in a condition were individual selection is low (structured condition). In contrast, with high individual competition (non-structured condition) there should be a return to basal virulence levels or even an increment in virulence[57,58,61].

The obtained results indicate that the difference between wild-type and mutant bacteria was only visible when taking the environment into consideration (structured and non-structured conditions). As expected, the environment had no effect on survival of animals fed on wild-type bacteria (Figure 10), since this population is expected to have lower levels of genetic diversity. In contrast, *C. elegans* fed on mutant bacteria in the structured condition had higher longevity (Figure 10), indicating that the environment had an impact on virulence levels of mutant bacteria. This result is in agreement with the hypothesis in which mutant populations should have lower virulence levels that should only be maintained where individual selection is low.

During the first survival assay with wild-type and mutant populations (Figure 10), bacteria were selected and the survival of individuals exposed to the selected bacteria was analyzed. Despite only being selected for a single round, this could be enough for populations to show signs of evolution, since fitness effects can be tenfold greater at the beginning of adaptation[88,90].

One way to demonstrate evolution is to compare ancestral and derived bacteria. Ancestral and derived wild-type bacteria were not different (Figure 10, 11A), which was expected since wild-type bacteria had low levels of genetic diversity. On the other hand, mutant ancestral bacteria were different from the derived ones (Figure 10, 11B).

This is expected since mutant bacteria probably have higher levels of genetic diversity, even though there were no differences between mutant and wild-type bacteria upon direct comparison. In contrast with previous findings, mutant bacteria selected in different environments were not different (Figure 11B). This result could be explained by the fact that, in non-structured conditions, all the different genotypes did not compete with each other, causing a delay in returning to basal virulence levels.

Even though differences in ancestral and derived mutant bacteria were found, bacteria selected in the three different time-points were not different (Figure 11). This indicates that differences in bacteria were not enough to induce differences in the time-points of selection.

5. Antibiotic resistance and trade-offs

In this work, it was found that *S. marcescens* is capable of gaining kanamycin resistant and this is associated with a trade-off in growth capacity and a possible one with tetracycline resistance (Figure 15). Previous works have shown that variation in colony morphology is common during adaptation[91]. One of the most frequently observed morphotypes are small-colony variants (SCVs), which were associated with higher resistant to aminoglycosides (kanamycin, gentamicin and streptomycin) and sensitivity to tetracycline during adaptation to macrophages[91].

In order to test the impact of these trade-offs in the interaction with *C. elegans*, survival of worms exposed to *S. marcescens* in NGM-kanamycin plates was analyzed and results showed that individuals in NGM-kanamycin plates had higher survival rates (Figure 16). This is in agreement with lower levels of bacterial proliferation due to a trade-off with antibiotic resistance.

6. Final remarks

During the course of this work, some setbacks had to be addressed throughout. There were several problems with contaminations, which highlighted the importance of properly controlled conditions when working with microorganisms. Obstacles regarding the initial protocols also arose, which brought to light the need and importance of suitable protocols and optimization.

Regardless, we fulfilled the majority of the initial aims along with the establishment of an efficient protocol to perform experimental evolution of gut microbiota capable of altering *C. elegans'* longevity. We demonstrated that the protocol works and results showed that, in a short period of time, it is possible to indirectly evolve gut bacteria, which in turn influences the longevity of its host. Moreover, even without a complete optimization, the structured and non-structured conditions are sufficient to create environments with different levels of selection that, in turn, are important for the evolutionary dynamics of bacteria.

All things considered, it will be extremely interesting to see to what extent gut bacteria are capable of altering host traits and to test if multilevel selection is important for bacterial evolution in general or if it is a specific process for host-microbiota interactions. Finally, it will be interesting not only to look at *C. elegans* lifespan, but also to *C. elegans* fertility, since a number of works have correlated gut bacteria with fertility problems[23]. Another feature that would be interesting to analyze is *C. elegans* healthspan, given that an increment in lifespan does not necessarily mean an increment in quality of life[92].

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Supplementary Information

S1 Survival assays

S1.1 Characterization of C. elegans lifespan in different bacteria

Survival of wild-type N2 and *hsf-1* mutants fed on different bacteria was performed according to the survival protocol, with the exception that worms were seeded as L1 and not as unhatched embryos and adults were individually transferred with a picker. Two different sets of experiments were performed: in the first set, small (15mm) NGM plates seeded with *E. coli* OP50, *E. coli* MG1655, *E. coli* IAI1, *E. coli aroD* and *S. marcescens* db10 were used. Bacteria were seeded in 10µl drops in the plates center and worms were transferred daily onto identical plates seeded with the same bacteria. The second set mimics the experimental evolution protocol, given that worms were allowed to feed for 5 hours in NGM plates (90mm) seeded with a lawn of each bacteria. After this, individuals were transferred onto small (15mm) NGM plates seeded with a central drop of *E. coli* OP50. In both experiments five replicates of each condition were used, each one with 10 individuals, totaling 50 individuals per treatment.

In results from the first set, in comparison with *E. coli aroD*, as expected N2 individuals (Figure S1A) fed on *S. marcescens* had the higher risk of death (*hazard ratio=3.377* and *p-value<0.0001*), followed by worms fed on *E. coli* IAI1 (*hazard ratio=1.727* and *p-value=0.015*). Finally, no differences were found in survival of N2 individuals fed on the remaining bacteria (p-*value=0.88*). For *hsf-1* mutants (Figure S1B), differences were only found in survival of individuals fed on *E. coli* OP50, which had the lower risk of death (*hazard ratio=0.443* and *p-value=0.0007*).

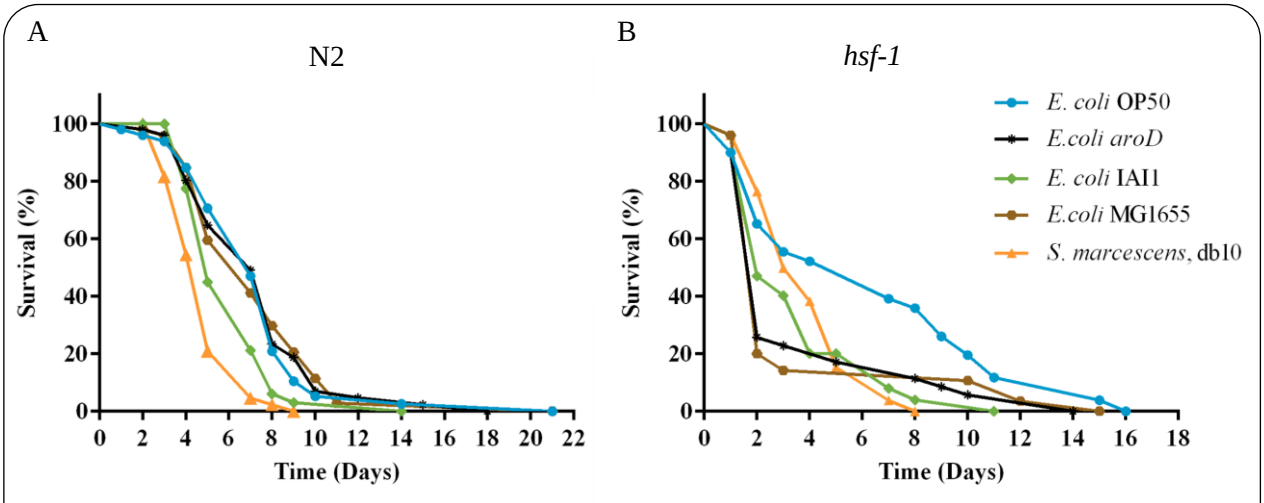

Figure S1 – Survival of *C. elegans* fed on different bacteria.

A – survival of wild-type N2 individuals; **B** – survival of *hsf-1* mutants; *C. elegans* fed on *E. coli* OP50 (*blue*), *E. coli aroD* (*black*), *E. coli* IAI1 (*green*), *E. coli* MG1655 (*brown*) and *S. marcescens* db10 (*orange*). 5 replicates per condition, n=10 in each condition.

In the second assay, just as before, wild-type N2 individuals (Figure S2A) did not present differences in survival when feeding on *E. coli* OP50 or *E. coli aroD* (*p-value=0.13*) and individuals fed on *S. marcescens* had the lower survival rate (*hazard ratio=2.034* and *p-value=0.001*). Once more, *hsf-1* mutants (Figure S2B) fed on *E. coli* OP50 had the highest survival rate (*hazard ratio=0.618* and *p-value=0.027*).

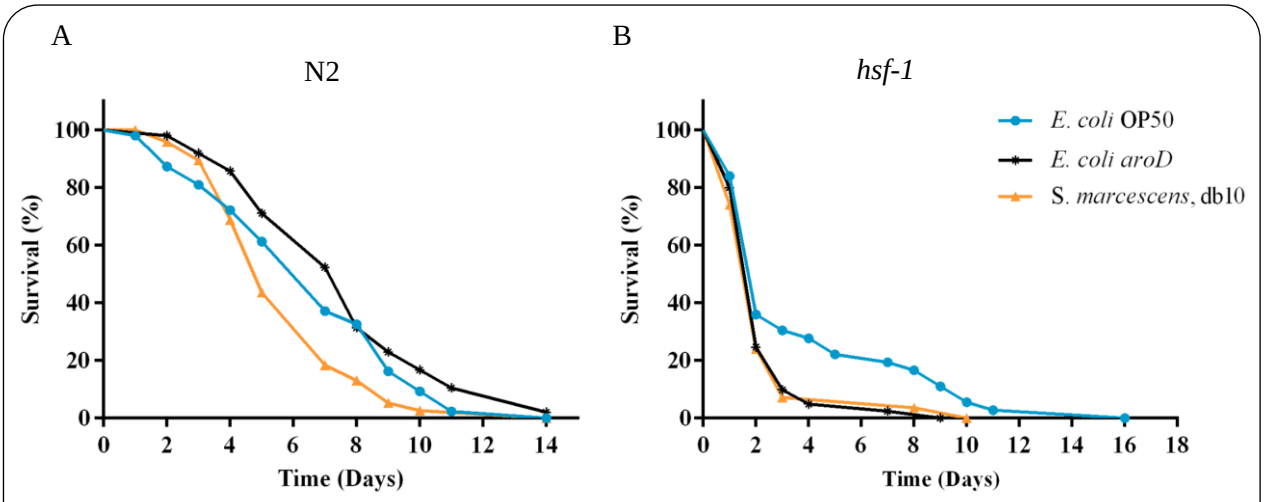

Figure S2 – Survival of *C. elegans* exposed to different bacteria.

A – survival of wild-type N2 individuals; **B** – survival of *hsf-1* mutants; *C. elegans* exposed over-night to *E. coli* OP50 (*blue*), *E. coli aroD* (*black*) and *S. marcescens* db10 (*orange*). 5 replicates per condition, n=10 in each condition.

S1.2 Transferring C. elegans with filters

During the development of the filtering protocol, different filter sizes were tested in order to optimize the size that allow the separation of adults from eggs, L1 and L2 larvae. The results are summarized in Table S1.

TableS1 – Retention for different filter sizes and ability of separating *C. elegans* adults from eggs, L1 or L2 larvae

Filter sizes	Eggs	L1	L2	Adults
30µm	Yes*	NA	NA	Yes
40µm	No	No	No	Yes
50µm	No	No	No	Yes**

*A portion of eggs was retained under the filter

** A portion of adults pass through the filter

NA – Not-applicable

To test the efficiency of the protocol, a survival assay with wild-type N2 and *hsf-1* mutants was performed (Figure S3). As expected, N2 individuals had lower risk of death than *hsf-1* mutants (*hazard ratio=0.515* and *p-value<0.0001*).

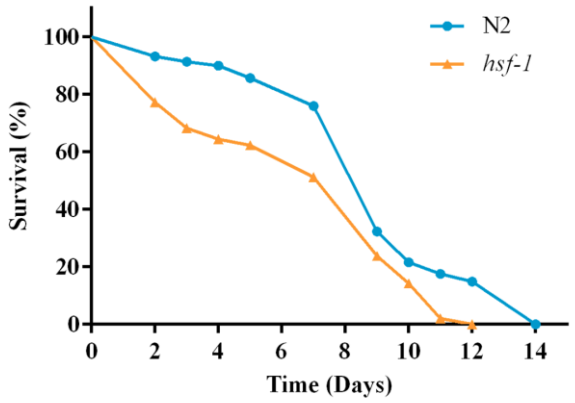

Figure S3 – Survival of *C. elegans* when transferred with filters.
Wild-type N2 n=716 (*blue*) and *hsf-1* mutant n=774 (*orange*).

S1.3 Survival of C. elegans with pathogenic bacteria

For the survival of *C. elegans* with pathogenic bacteria, wild-type N2 and *hsf-1* mutants were fed over-night on *E. coli* IAI1, *E. coli* IAI1 mCherry and S. *marcescens* db10. NGM plates were used with one bacterial strain per plate and one replicate per bacteria. The following day, individuals were transferred onto NGM plates seeded with *E. coli* OP50 mCherry (individuals that fed on *E. coli* IAI1 mCherry were transferred to *E. coli* OP50 without fluorescence).

S1.4 Survival of hsf-1 mutants in ancestral and derived S. marcescens

At the end of the fourth round of selection in the experimental evolution assay, a survival assay with *hsf-1* mutants fed on ancestral and derived *S. marcescens* was done. Worms were exposed to bacteria in NGM-tetracycline plates (150mm) and then transferred onto NGM plates (90mm) seeded with *E. coli* OP50 mCherry, where they were maintained. Each condition was replicated two times. There was no difference between individuals fed on ancestral or derived *S. marcescens* (*p-value=1*) (Figure S4).

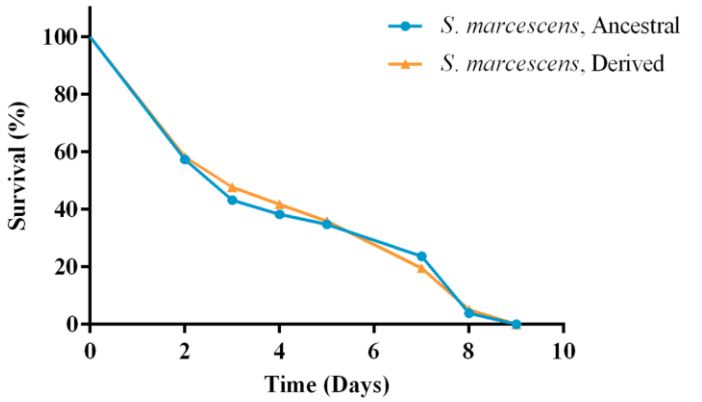

Figure S4 – Survival of *C. elegans* fed on *S marcescens*.

Ancestral *S. marcescens* (*blue*) n=538; Derived *S. marcescens* (*orange*) n=666; each condition was replicated 2 times.

S1.5 Survival of C. elegans in a mixture of bacteria

Survival of N2 individuals fed on a mixture of *S. marcescens* db10 and db11 was performed in structured and non-structured conditions. L4 individuals were transferred onto to 150mm NGM-tetracycline plates, previously seeded with a mixture of individual colonies of both *S. marcescens* db10 and db11. OD_{600} of the over-night culture was measured and corrected in order to guarantee that both bacteria were seeded in equal proportions. After an over-night, worms were transferred and maintained in PFN plates seeded with *E. coli* OP50 mCherry.

Results show that one individual from day 4 of the structured condition presented a lower frequency for *S. marcescens* db10 (Table S2 – individual highlighted in red).

TableS2 – Frequency of *S. marcescens* in two different conditions (non-structure – NS and structure – S) at different time-points.

Condition										
Time	**NS**					**S**				
2	0,924	0,976	0,895	0,942	0,985	0,980	0,935	0,999	0,864	0,633
4	0,990	0,995	0,989	0,995	0,994	0,995	0,982	0,995	**0,150**	0,988
6	0,96	0,980	0,911	0,995	0,989	0,998	0,981	0,985	0,995	0,997
8	0,975	0,990	0,989	0,975	0,972	0,984	0,990	0,980	0,968	0,965

The particular individual was excluded from the data and the analysis was run once more and results showed that without this particular individual, *S. marcescens* db10 started at equal proportions in the structured and non-structured conditions (*p-value=0.166*) (Figure S5).

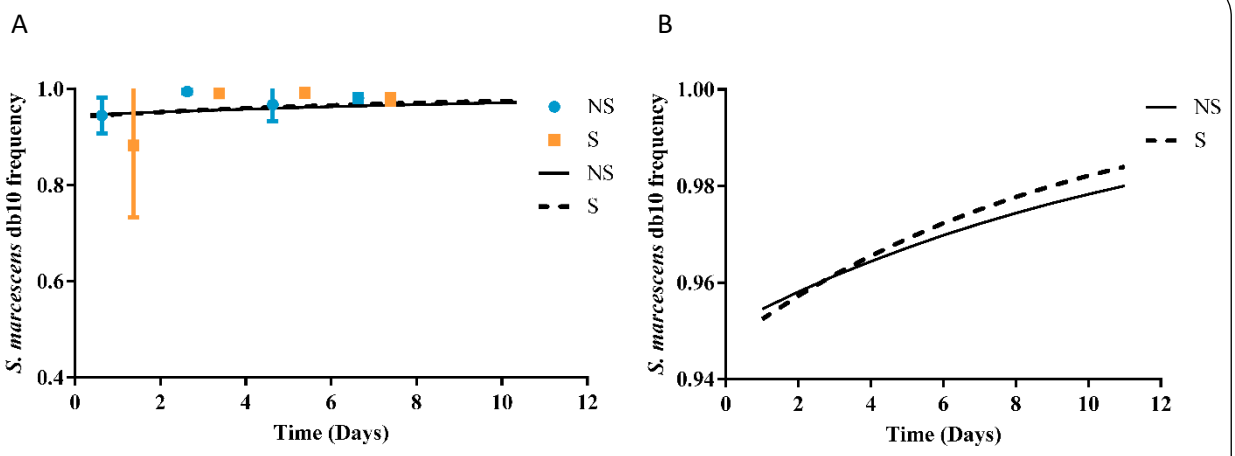

Figure S5 – Frequency of *S. marcescens* db10 inside *C. elegans'* gut without a particular individual.
A - Frequency of recovered bacteria at different time-points and conditions: Non-Structure (*blue*) and Structure (*orange*). Each point represents mean value for frequency calculated from 5 CFUs measures for *S. marcescens* db10 and db11. Vertical bars represent standard deviation (SD). Black lines represent predicted model for non-structured (*full*) and structured (*dashed*) conditions. **B –** Enlargement of predicted model from A; non-structured (*full*) and structured (*dashed*) conditions.

S2 Mutagenesis

To increase genetic diversity in the experimental *S. marcescens* populations, a mutagenic protocol was performed. The protocol followed one described in Parkhomchuk *et al.* (2009), where a *E. coli* K-12 CC102 strain was used. The authors found that on average, mutant bacteria had 70 mutations per genome and the majority of changes were G:C to A:T transitions, associated with 50-60% survival rate.

Results show that the effect of EMS on viability was more pronounced for *E. coli* OP50 (Figure S6A), since at 15 minutes *E. coli* OP50 had 15% of survival comparing to *S. marcescens* which had 55% survival at the same time-point (Figure S6B). 60-minutes results are not shown because there was a bacterial lawn, making it impossible to count individual colonies. *S. marcescens* populations from the 15 minutes of mutagenesis were used, since survival was 55% and it is possible to predict the expected number of mutations in the genome.

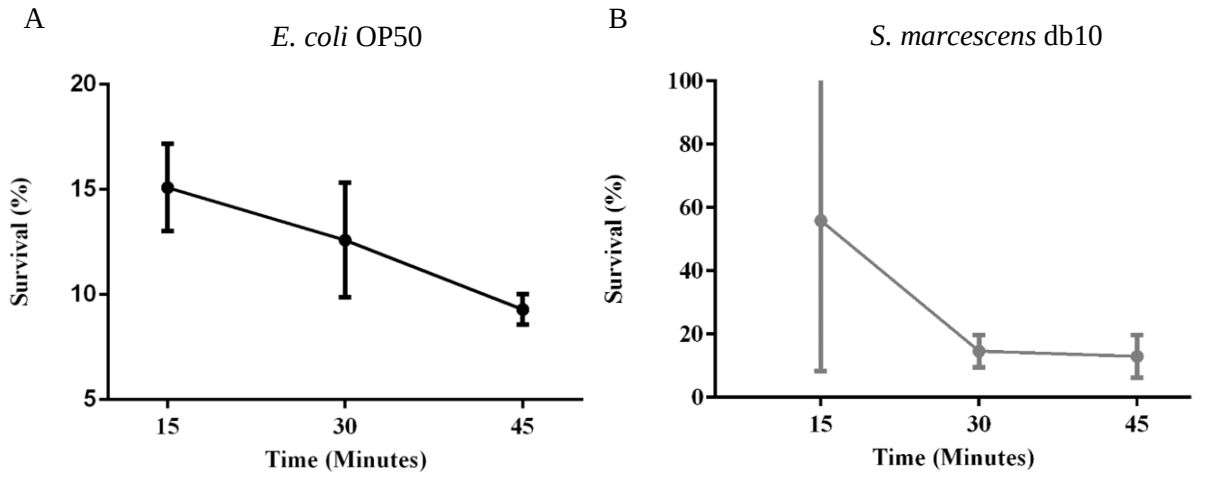

Figure S6 – Viability of bacteria exposed to EMS.

Survival of bacteria when exposed to EMS at different time-points (15, 30 and 45 minutes). **A** – survival of *E. coli* OP50; **B** – survival of *S. marcescens*. Vertical bar represents standard deviation (SD).

S3 Different levels of selection

S3.1 Proportion of C. elegans

In a first approach to create experimental conditions that allow different levels of selection, different proportions of *C. elegans* feeding on bacteria were used. The main idea of the protocol was to create a condition where the ratio between the number of *C. elegans* and the number of bacterial colonies was approximately one. In this condition, each worm should only have the opportunity to feed on one bacterial colony, leading to only one bacterial genotype inside each individual, thus reducing individual competition between bacteria. On the other hand, when the ratio is inferior to one, each individual can feed on more than one bacterial colony, thus increasing the number of different genotypes inside each individual and consequently increasing the levels of bacterial individual selection.

In order to implement this concept, either 50 or 500 worms fed on both *E. coli* OP50 mCherry and GFP were used. Bacteria were seeded in NGM plates (90mm) at equal proportions and diluted in order to obtain approximately 1000 individual bacterial colonies. As a control, plates seeded with only *E. coli* mCherry or GFP were used. In each petri dish, 50 or 500 L4 individuals were placed by liquid drops where they explored the environment and fed on the bacteria for 5 hours. After 5 hours, in order to clean external bacteria, 10 individuals were transferred onto plates with LB-ampicillin where they remained for 5 minutes.

After that, individuals were crushed in 5µl of M9 buffer, diluted and the recovered solution was plated in 10µl drops in LB in order to study the fluorescence of bacteria inside *C. elegans'* gut.

The same protocol was applied in a new experiment, where 90mm and 150mm petri dishes were used as a way to increase the distance between each bacterial colony and subsequently decrease the probability of an individual feeding on two different bacterial colonies.

Results show the majority of individuals had a mixture of *E. coli* mCherry and GFP inside the gut (Figure S7, S8), indicating that each individual fed on more than one colony.

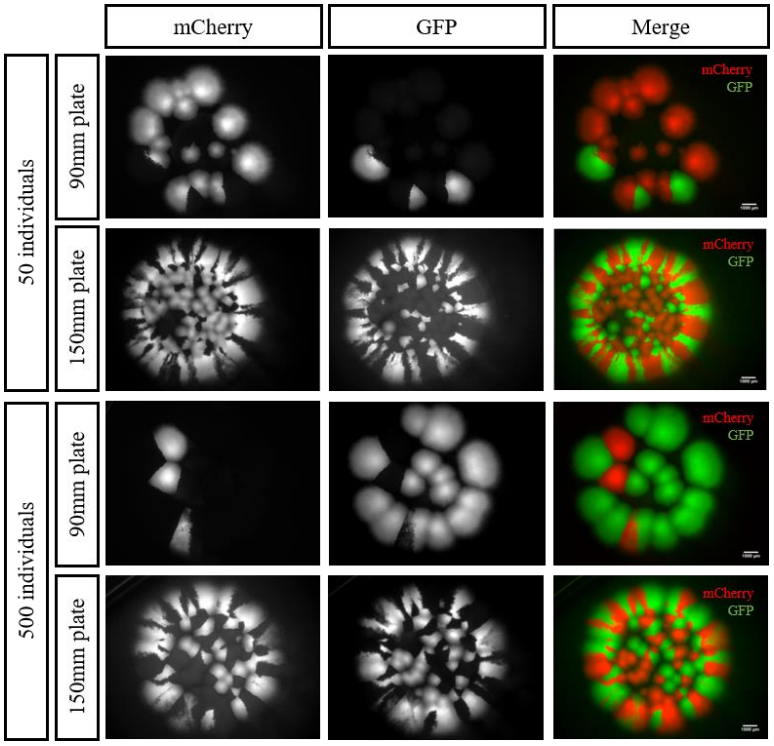

Figure S7 – Recovered gut bacteria.

Bacteria were recovered from individuals fed on *E. coli* OP50 mCherry and GFP in 90mm or 150mm plates. Two conditions were used, one with 50 individuals and another with 500 individuals. First column mCherry fluorescence, second column GFP fluorescence and third column merge. Scale bar=1mm.

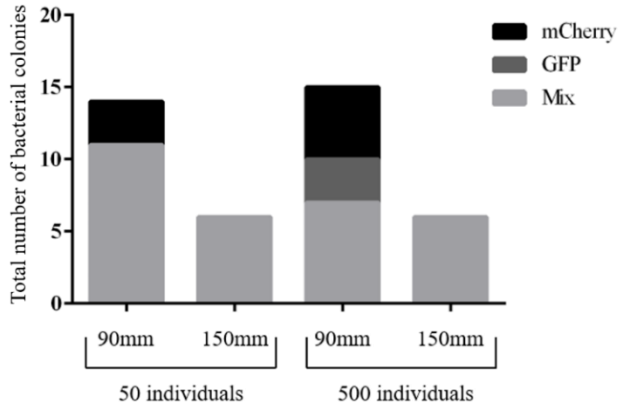

Figure S8– Total number of recovered gut bacteria.

Bacteria were recovered from individuals fed on *E. coli* OP50 mCherry and GFP in 90mm or 150mm plates. Two conditions were used, one with 50 individuals and another with 500 individuals. Number of mCherry colonies (*black*), number of GFP colonies (*dark grey*) and number of mCherry and GFP colonies (*light grey*). 90mm plate condition was replicated two times and 150mm plate condition one time.

S3.2 3D scaffold

In a second approach, a honeycomb-like 3D scaffold composed by 594 individual cells was used. To test the efficiency of the bacteria placing protocol, the number of individual colonies inside each unit was analyzed by counting the number of bacterial cells inside each cell in two different plates. In the first plate, 67 cells were counted and in the second one, 64 cells.

Results showed the majority of cells had only one bacterial colony (Figure S9). However, a considerable number of cells without colonies or with 2-4 bacterial colonies was also found.

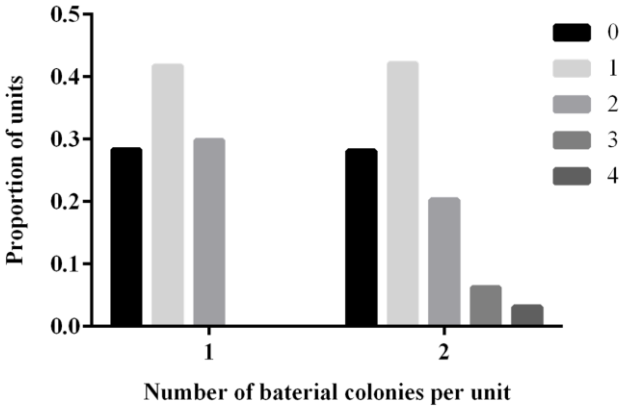

Figure S9 – Proportion of units with different numbers of bacterial colonies.

Two plates were counted; 67 individual units in the first one and 64 in the second one.

S4 Experimental Evolution

For the first experimental evolution protocol, wild-type N2 and *hsf-1* mutants were exposed to mutant *S. marcescens* db10 in the structured and non-structured conditions and selected in the three described time-points (T1, T2 and T3). Each condition was replicated three times, totaling 36 experimental populations. With this protocol, four rounds of selection were performed (approximately three months).

In more detail, in the first round of selection, after being exposed to *S. marcescens*, individuals were transferred with M9 buffer onto NGM plates (90mm) seeded with *E. coli* OP50 mCherry. In the second and third rounds of selection, individuals were transferred with M9 buffer supplemented with kanamycin and plated into plates without antibiotic (NGM seeded with *E. coli* OP50 mCherry). Finally, in the fourth round, individuals were transferred in M9 buffer (once again without antibiotic) but were placed in plates with NGM-kanamycin and seeded with *E. coli* OP50 mCherry (kanamycin resistance).

Contrary to the final experimental evolution, the survival of individuals was not analyzed during this experiment. The time-points of selection were calculated based on a previous survival curve with *S. marcescens* and took into consideration the total number of missing individuals overtime.

Just as in our final protocol, the T1 and T2 selected bacteria were re-selected and plated in LB-tetracycline. This process was done with a replicate plate and filter paper that allowed us to transpose one plate to another.

S5 Kanamycin resistance in *S. marcescens*

To confirm the ability of *S. marcescens* db10 to gain kanamycin resistance, the bacteria was grown in liquid culture of LB and LB-kanamycin at three different concentrations: 50µg/ml (normal concentration), 25µg/ml and 12,5µg/ml. LB-ampicillin (100µg/ml) and LB-gentamicin (10µg/ml) were used as controls. Bacteria were incubated over-night at 37°C with shaking.

The following day, 100µl of bacterial growth from the 3 different kanamycin concentrations was plated in LB, LB-tetracycline and LB-kanamycin plates. These plates were then incubated over-night at 37°C. In parallel, 100µl of bacterial growth from the 25µg/ml kanamycin liquid culture was recovered and transferred into 10ml of liquid LB supplemented with either 50µg/ml or 25µg/ml kanamycin. The parallel cultures were incubated over-night at 37°C with shaking.

On the third day, a portion of bacterial colonies from the tetracycline plates was recovered, and re-plated in LB, LB-tetracycline and LB-kanamycin plates. As a control, stock *S. marcescens* db10 plated in LB, LB-tetracycline and LB-kanamycin was used and incubated for 48 hours at either 37°C or at 20°C.

S6 Influence of bacteria competition in bacteria virulence

Individuals fed on a mixture of *S. marcescens* db10 and d11 were selected in four different time-points in order to characterize the presence and dynamics of the two strains inside the gut. Five individuals were selected at random and crushed according to the experimental evolution time-points and protocol. An extra time-point was added - T0 – which corresponds to the first day after being transferred to *E. coli* OP50 mCherry. Recovered bacteria were plated in LB, LB-tetracycline and LB-tetracycline-streptomycin (two replicates per individual). Bacteria were not plated in drops, but rather seeded in order to form individual colonies scattered around the plate. CFU analysis was performed 48 hours after seeding.

The growth of the two bacteria when plated together or individually was also analyzed. Bacteria were plated in LB, LB-tetracycline and LB-tetracycline-streptomycin. CFUs were analyzed after growing over-night at 37°C.

S7 Statistical analysis and graphics

All the statistical analysis was performed in R, version 3.3.1. Statistical analysis of the results from section 4.1 of the Results was performed with a generalized linear model given by the *glm* function in R. This function transforms a linear predictor and error distribution into a log odds scale. In Figure 14, the predicted model was calculated based on real data using the *glm* function. The variance analysis was performed using the *var.test* function in R. This function performs an F test to compare the variances of two samples. Finally, all graphics present in this work were made using GraphPad Prism 6 and images were analyzed using FIJI software.